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GRANT NUMBER DAMD17-96-1-6016

Title: An Immunotherapeutic Approach to the Treatment and Prevention of Breast Cancer, Based on Epidermal Growth Factor Receptor Variant, Type III

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REPORT DATE: May 1999

TYPE OF REPORT: Final

PREPARED FOR: Commander  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

19991208 170

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# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 1999	3. REPORT TYPE AND DATES COVERED Final (1 Jul 96 - 31 May 99)	
4. TITLE AND SUBTITLE An Immunotherapeutic Approach to the Treatment and Prevention of Breast Cancer, Based on Epidermal Growth Factor Receptor Variant, Type III			5. FUNDING NUMBERS DAMD17-96-1-6016	
6. AUTHOR(S) Lisa K. Gilliam Dr. Laura Hale				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Medical Center Durham, NC 27710			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200)  Micrometastasis of breast cancers prior to detection and surgical resection often limits the success of current cancer therapies. New methods to treat and to prevent metastatic breast cancer are thus urgently needed. Treatments based upon immunotherapy have recently gained support, due to the high specificity of immune recognition and the resultant potential for decreased complications related to treatment. This project has focused on development of reagents for passive immunotherapy of breast cancer, based on a variant of the epidermal growth factor receptor (EGFRvIII) found in over 27% of breast cancers. We have genetically engineered constructs for an antibody bispecific for EGFRvIII and the CD3ε T cell activation antigen. This bispecific antibody should be capable of redirecting the cytotoxic response of T cells with a broad range of specificities against breast cancer cells that express EGFRvIII. We have also constructed an adenovirus that efficiently transduces the EGFRvIII gene into a variety of target cells to facilitate <i>in vitro</i> testing of the bispecific antibody. These reagents are available to researchers interested in further testing and characterization of this promising passive immunotherapy approach.				
14. SUBJECT TERMS Breast cancer Epidermal growth factor receptor Immunotherapy		Bispecific antibody Cytotoxicity Antigen presentation		15. NUMBER OF PAGES 239
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		16. PRICE CODE
19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABSTRACT Unlimited		

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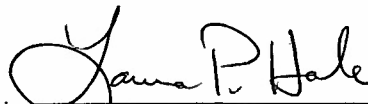
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(bound copy of L.K. Gilliam dissertation also included)



## Introduction

The goal of this project is to develop new strategies for immune-based therapy of breast cancer, based on expression of the tumor-associated antigen EGFRvIII, a variant of the epidermal growth factor receptor. EGFRvIII was detectable by immunohistochemistry in 3/11 breast carcinomas tested, with mRNA corresponding to EGFRvIII detectable in these 3 plus an additional 5 (total 8/11) by RT-PCR and ethidium bromide staining (Wikstrand 1995). Flow cytometric analysis demonstrated cell surface reactivity of tumor cells with anti-EGFRvIII monoclonal antibodies (mAbs) in 5 of 5 mechanically dissociated primary breast carcinomas. EGFRvIII is not detected in most normal tissues. Thus EGFRvIII is a tumor-associated that may be a useful target for immunotherapy. Activation of cytotoxic T cells to carry out their cytolytic function normally requires engagement of their specific T cell receptor by appropriate antigenic peptides on the target cell in the context of MHC Class I molecules, plus additional activation signals. We proposed to bypass the need for specific antigenic recognition by using an antibody that can specifically recognize the EGFRvIII antigen on the tumor target cell and also activate T cells directly. This can be accomplished by creating antibodies with different antigen binding sites on each of their two antigen binding sites. Such antibodies are termed bispecific antibodies. An antibody bispecific for the EGFRvIII tumor antigen and for the CD3 $\epsilon$  T cell activation molecule can thus bring tumor cells in close proximity to cytotoxic T cells and also activate their cytolytic function. An advantage of this approach is that any cytotoxic T cell would thus be capable of lysing the tumor target, regardless of its innate T cell receptor specificity. The use of passively administered bispecific antibodies does not require the generation of *de novo* immune responses, making it compatible with other cancer treatments that may decrease immune competence in treated patients.

## Body

This project was part of the doctoral research of Lisa K. Gilliam, in partial fulfillment of requirements for the Ph.D. degree in pathology. Dr. Gilliam completed her Ph.D. work in August 1998, then returned to medical school to finish requirements for her M.D. degree. She was awarded the Ph.D. degree in December 1998 and the M.D. degree in May 1999. Dr. Gilliam will begin her residency in internal medicine at University of Washington in Seattle on July 1, 1999, in preparation for a career in hematology/oncology and clinical research in breast cancer.

The technical objectives for this proposal have been met as detailed below. The original technical objectives were as follows:

- 1) The expression pattern of the EGFRvIII tumor antigen, including tumor specificity and prevalence, will be determined for a large panel of specimens from breast cancer patients, for established breast cancer cell lines, and for a broad spectrum of normal adult and fetal tissues, using RT-PCR, flow cytometry, immunohistochemistry, and Western blot assays.

Results showed that EGFRvIII is relatively specific for tumor tissues, with absence of this variant protein in most normal tissues. However, studies showed low level expression of EGFRvIII in some fetal tissues as well as in adult human thymocytes. The expression of EGFRvIII is much higher in tumors than in thymocytes and the contribution of adult thymopoiesis to the T cell repertoire is likely small (Haynes 1999a; Haynes 1999b). Thus this antigen is still potentially useful for passive immunotherapy. All breast cancer cell lines tested lacked expression of EGFRvIII. This is not unexpected, since brain tumors that strongly express EGFRvIII *in vivo* progressively lose expression of EGFRvIII when cultured *in vitro*. However EGFRvIII expression of brain tumors can be maintained by growth as murine xenografts. These results suggest that although EGFRvIII may contribute to tumor growth *in vivo*, it is detrimental to *in vitro* growth of tumor cells. These results triggered the development of an adenovirus transducing EGFRvIII cDNA to facilitate creation of EGFRvIII-expressing cells to be used in functional assays and tests of bispecific antibody efficacy (see below).

- 2) The existence of a naturally occurring anti-EGFRvIII immune response in breast cancer patients as well as the ability to generate a humoral or cellular immune response against EGFRvIII in these patients will be tested *in vitro*, using enzyme immunoassays of patients' serum and cytotoxicity of patients' TILs and PBLs against antigen presenting cells loaded with EGFRvIII-specific peptides.

The unanticipated discovery of expression of EGFRvIII in adult thymocytes prompted replacement of this original technical objective with a revised objective, as detailed in the 1998 progress report.

- 3) An antibody bispecific for both EGFRvIII and CD3 $\epsilon$  will be constructed to investigate this bispecific construct as a means to bypass requirements for specific T cell receptor/peptide-MHC matching and to direct CTLs to kill tumor cells regardless of their natural specificity.

Molecular constructs encoding heavy and light chains of EGFRvIII and CD3 $\epsilon$ -specific mAbs were constructed and modified to facilitate combination as bispecific antibodies, using fos and jun zipper peptides as proposed. These constructs were sequenced and can now be used for large-scale production of bispecific antibodies. As mentioned earlier, one problem with determining the efficacy of bispecific antibodies produced from these constructs *in vitro* is the lack of stable EGFRvIII-expressing breast cancer cell lines. Therefore, an adenovirus that efficiently transduces EGFRvIII into breast cancer cells was constructed and characterized. These reagents are available to any researcher interested in further pursuing the use of this approach in pre-clinical or clinical trials. The adenovirus may also be useful independently as a vaccine to generate active immunity against EGFRvIII.

- 4) Immunological reagents will be developed for the inherited breast cancer susceptibility gene BRCA2. These will be used to study the expression pattern of BRCA2 protein in various tissues, as well as its intracellular localization, and to gain clues regarding the normal function of this gene product.

This technical objective was substituted for the original technical objective 2 as outlined above and described in detail in the 1998 progress report. A monoclonal antibody specific for BRCA2 sequences was developed. Although this mAb detects full-length BRCA2 in immunoprecipitation assays followed by Western blotting, it generally does not react with most cells or tissues in direct Western blotting or immunohistochemical assays. We feel that this is due to the low prevalence of BRCA2 protein in most cells and tissues, that allows detection only after antigen concentration as occurs in the immunoprecipitation/Western blot protocol. However, we unexpectedly found reactivity with our anti-BRCA2 mAb 2C9 in terminally differentiating normal squamous epithelial cells and in well-differentiated squamous cell carcinomas. The specificity and significance of this finding is still unclear.

Details of all these studies are contained in the Ph.D. dissertation of Dr. Lisa Gilliam, submitted as an addendum to this report.

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- Haynes, B.F., Hale, L.P.: Thymic function, aging, and AIDS. Hospital Practice, 34:59-88, 1999.

## **Key Research Accomplishments**

- Determined expression pattern of EGFRvIII tumor-associated antigen in normal and malignant tissues.
- Created molecular constructs necessary to generate an bispecific antibody recognizing both the EGFRvIII and CD3 $\epsilon$  antigens.
- Created an adenovirus that efficiently transduces EGFRvIII cDNA and generates high level expression of EGFRvIII on infected cells.
- Generated and characterized the reactivity of a monoclonal antibody (2C9) specific for BRCA2 sequences.

## **Reportable Outcomes**

### Manuscripts, Abstracts, and Presentations

Gilliam, L.K. Immune Based Approaches to Breast Cancer Susceptibility and Treatment, Ph.D. dissertation, Duke University, 1998.

Gilliam, L.K., Scarce, R.M., Cirisano, F.D., Lobenhofer, E., Marks, J.R., Hale, L.P.: Production of a monoclonal antibody reactive with BRCA2 protein sequences. Proceedings of the American Association for Cancer Research, 38:243, 1997.

### Degrees Supported by this Award

Lisa K. Gilliam, Ph. D. in pathology, Duke University, December 1998.

### Development of Cell Lines, Tissue, or Serum Repositories

2C9 hybridoma, specific for BRCA2 sequences (exon 11)

molecular constructs for bispecific antibody:

anti-EGFRvIII L8A4 heavy chain-jun

anti-EGFRvIII L8A4 light chain

anti-CD3 $\epsilon$  OKT3 heavy chain-fos

anti-CD3 $\epsilon$  OKT3 light chain

adenovirus transducing EGFRvIII under control of CMV IE promoter

### Employment Applied for and Received as a Result of this Award

Lisa K. Gilliam, M.D. Ph.D., intern and residency position in internal medicine at University of Washington, Seattle. July 1999 - June 2002.

**#1631 Ceruloplasmin expression is reduced during peroxisome proliferator-induced hepatocarcinogenesis.** Teepe, A.G., Elm, M.S., Epley, M.J., Shinozuka, H., Rao, K.N., and Eagon, P.K. VA Medical Center, Pittsburgh, PA 15240, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Ceruloplasmin (CP), a serum  $\alpha_2$ -globulin synthesized by the liver, has a number of important functions. In addition to being an acute-phase reactant protein, it possesses oxidase activity for ferrous ions and aromatic amines, as well as free radical scavenger activity. The serum level of this critical protein is markedly reduced in Wilson's disease, as a result of a defective P-type ATPase (WD gene) involved in insertion of copper into the CP protein. Long-Evans Cinnamon rats exhibit a homologous defect; it is of note that this strain of rats is highly susceptible to development of hepatocellular carcinomas (HCC). In other studies, elevated CP levels have been reported in several types of cancer and inflammation, as well as during pregnancy and exposure to estrogens. Thus, we tested whether CP serum levels and steady-state hepatic CP and WD gene mRNA levels are altered in a rat model of nongenotoxic hepatocarcinogenesis. Male Fischer 344 rats were fed diets containing the peroxisome proliferators diethylhexylphthalate [DEHP] or clofibrate [CLF] for 3-60 days to induce hepatic hyperplasia, and 4-chloro-6-(2,3 xylidino)-2-pyrimidinyl-thio(N- $\beta$ -hydroxyethyl) acetamide [BR931] for 10 months to induce HCCs. All BR931-treated rats developed multiple tumors, which were analyzed separately from surrounding liver. Exposure to DEHP and CLF (3, 7 & 60 days) resulted in an immediate and sustained decrease in serum CP oxidase activity ( $p < 0.05$  compared to age-matched control rats). This decrease was mirrored by reductions in both hepatic CP and WD gene steady-state mRNA levels (50% and 35%, respectively). Tumor-bearing rats also had significantly lower serum CP oxidase activity than age-matched controls ( $p < 0.05$ ). In the tumors, the steady-state CP mRNA level was reduced (49%) as compared to control liver ( $p < 0.05$ ), and was reduced in liver surrounding the tumors ( $p < 0.05$ ). The observed reductions of CP and WD gene mRNA levels in livers of treated rats provide an explanation for the reduced CP activity in serum of these rats. The consequence of this reduced expression of CP may be a loss of oxidase and free radical scavenger capacity in the tumor-bearing rats, which may contribute to both liver damage and the carcinogenic process.

**#1632 p53 and proliferating cell nuclear antigen (PCNA), and genomic instability in premalignant oral lesions.** Shin, D.M., Kim, J., El-Naggar, A., Lee, J.J., Lippman, S.M., Lee, J.S., Hong, W.K., and Hittelman, W.N. The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

To better understand the pathobiology of premalignant oral lesions, the selected biomarkers were studied in 68 premalignant tissue biopsies derived from 59 patients who were enrolled in a prospective randomized chemopreventive study. Baseline tissue samples consist of 53 hyperplasia (HYP), 11 mild dysplasia (DYP1), 3 moderate dysplasia (DYP2), and 1 severe dysplasia (DYP3). p53 and PCNA expression was analyzed by immunohistochemistry and numerical chromosome alteration was evaluated by *in situ* hybridization using chromosome 9 centromeric DNA probe. p53 and PCNA expression was assessed by labeling index (positively expressed cells/total cells counted). Polysomy 9 (Pol 9) was defined by % of cells containing 3 or more chromosome copies. Results are tabulated as follows:

	HYP (n=53)	DYP1 (n=11)	DYP2 (n=3)	DYP3 (n=1)	P Value*
p53	0.09 $\pm$ 0.14	0.21 $\pm$ 0.22	0.23 $\pm$ 0.37	0.018	0.12
PCNA	0.16 $\pm$ 0.12	0.23 $\pm$ 0.13	0.22 $\pm$ 0.08	0.36	0.02
Pol 9	1.6 $\pm$ 1.6 (%)	2.8 $\pm$ 3.0 (%)	2.8 $\pm$ 0.7 (%)	3.1 (%)	0.04

\*Wilcoxon rank-paired test: HYP vs DYP1, DYP2, and DYP3.

p53 and chromosome 9 polysomy showed a moderate correlation (Pearson's coefficient = 0.43), whereas no significant correlation was found between p53 and PCNA or PCNA and Pol 9. This study suggests that expression of p53 and PCNA, and genomic instability manifested by polysomy increased with histologic progression in a multistep fashion in premalignant oral lesions, and p53 alterations may be associated with genomic instability in oral carcinogenesis.

**#1633 Chemotherapeutic effect on mutation frequency of *hprt* and T-cell receptor gene in pediatric patients with malignancy.** Sawada, M., Kubota, M., Akiyama, Y., and Furusho, K. Department of Pediatrics, Faculty of Medicine, Kyoto University, Kyoto, Japan 606

We previously reported pediatric ALL patients had high mutation frequency (MF) of *hprt* (*hprt*-MF) and of T-cell receptor gene (TCR-MF) after completion of therapy. In this study, we further investigated *hprt*-MF and TCR-MF of 28 pediatric patients with malignancy at diagnosis, including 2 patients with second malignancy, and serially examined 12 patients during chemotherapy in order to detect the influence of each chemotherapy on MF. The results are, (1) 2 out of 4 patients diagnosed Hodgkin's disease and 2 patients with second malignancy had high *hprt*-MF ( $4.9-9.8 \times 10^{-6}$ ) at diagnosis. The geometric mean value was  $1.1(\pm 0.2) \times 10^{-6}$  for the healthy controls. (2) Both *hprt*-MF and TCR-MF became elevated through chemotherapy, but they did not correlate well with doses of chemotherapeutic agents except alkylating agents that increased *hprt*-MF. Although the number of samples is limited, *hprt*-MF is considered to be a more sensitive assay to detect the genetic influence of chemotherapy.

**#1634 Trisomy 20 in bronchial epithelial cells from lung cancer patients and smokers.** Neft, R., Gilliland, F., Crowell, R., Bellinsky, S., and Lechner, J. Inhalation Toxicology Research Institute, Albuquerque, NM 87185, University of New Mexico, Albuquerque, NM 87131

Despite intensive efforts, the mortality rate for lung cancer (LC) is still close to 90%. A valuable approach to the early detection of LC might be to assay bronchial epithelial cells (BECs) for several chromosome aberrations associated with LC, such as trisomy 7 and 20. BECs were obtained from four sites in the lung following bronchoscopy and grown in cell culture. The presence of trisomy 20 was assayed by fluorescence *in situ* hybridization (FISH) in BECs using a directly labeled probe for 20q13.2. One LC patient with squamous cell carcinoma (SCC) showed trisomy 20 in one site, and a second patient with SCC exhibited trisomy 20 in two sites. A smoker, without LC, whose smoking history was 24 pack years, was also positive for trisomy 20 in two sites. In a previous study, two of these patients also had BECs positive for trisomy 7. None of the three patients had sites that were positive for trisomy 2 which is not associated with LC. These results show that FISH for chromosome 7 and 20 may prove useful as part of a battery of tests for the early detection of LC. (Research sponsored by US DOE/OHER, Contract DE-AC04-76-EV01013 and NIH SPOR Grant, Contract 1-P50-CA8184, Johns Hopkins University.)

**#1635 Alterations in the Cyclin/CDK/retinoblastoma pathway in early human breast tumor development.** Robles, A.I., Zenklusen, J.C., Lubet, R.A., Kellog, G.J., Klein-Szanto, A.J.P., and Conti, C.J. LHC, National Cancer Institute, Bethesda, MD 20892, National Center for Human Genome Research, Bethesda, MD 20892, DCP, National Cancer Institute, Bethesda, MD 20892, Fox Chase Cancer Center, Philadelphia, PA 19111, The University of Texas M.D. Anderson Cancer Center, Smithville, TX 78957

Recent studies have shown that overexpression and/or amplification of Cyclin D1 is a common event in invasive breast tumors. To determine if this is also the case in the premalignant lesions (ductal carcinoma in situ, DCIS) and which role do this alteration play in the cell cycle of the affected cells, we analyzed forty-six cases of human DCIS paired with invasive carcinomas and their distant metastasis for Cyclin D1 overexpression/amplification, as well as *Rb* and *p16* allelic loss. DNA was extracted from paraffin sections of all the lesions as well as from the normal surrounding tissue. Assessment of the amplification of Cyclin D1 was performed by differential PCR amplification respect to the control gene ( $\gamma$  IFN). Two regions of the genome have been analyzed by PCR for LOH incidence in these lesions: 9p21 (*p16*) and 13q14 (*Rb*). Overexpression of Cyclin D1 and loss of *Rb* were assessed using immunohistochemical staining with the appropriate antibodies. The results obtained indicate that, as in the invasive tumors, overexpression of Cyclin D1 is a common event in DCIS, while little LOH and loss of expression can be seen in both *Rb* and *p16*, indicating that the abrogation of their function occurs via the Cyclin D1 alteration. Supported by NCI Contract NO1 CN55185.

**#1636 Production of a monoclonal antibody reactive with BRCA2 protein sequences.** Gilliam, L.K., Searce, R.M., Cirisano, F.D., Lobenhofer, E., Marks, J.R., and Hale, L.P. Duke University, Durham, NC 27710

In order to study the protein product of the BRCA2 gene implicated in a subset of hereditary breast cancers, we have generated a monoclonal antibody using a bacterially-produced 46 kDa immunogen (termed BAC2) derived from exon 11 of the BRCA2 gene. Mab 2C9 is strongly reactive with both the purified 46 kDa BAC2 protein as well as with a 79 kDa BAC2-GST fusion protein from bacterial lysates in Western blot analysis, but is non-reactive with induced and uninduced lysates from bacteria expressing a non-overlapping portion of the BRCA2 protein. 2C9 detects a faint band compatible with the 384 kDa predicted MW of BRCA2 using immunoprecipitation of  $5 \times 10^6$  MCF-7 breast carcinoma cells and very sensitive detection conditions. No reactivity can be detected with 2C9 mAb using standard immunoblots, immunohistochemistry or flow cytometry of permeabilized cells, suggesting that the BRCA2 protein is present in cells at extremely low concentrations. Although further characterization is needed, this mAb will likely be a useful tool for determination of the presence and function of the BRCA2 protein.

**#1637 Rare Hras1 VNTR alleles in Spanish lung and lymphoid cancer patients.** Pifarre, A., Sánchez-Céspedes, M., Aldea, A.I., Calvo, R., Moreno, I., Vaquero, M., Monzó, M., and Rosell, R. Molecular Biology Laboratory of Cancer, Medical Oncology, Pathology and Hematology Services, University Hospital Germans Trias i Pujol, 08916 Badalona, Spain

We investigated the influence of rare alleles at the minisatellite region flanking the Hras1 gene, as well as associated increased lung and lymphoid cancer risk. We studied 200 Hras1 alleles from 100 unaffected controls, 96 alleles from 48 resected lung cancer patients and 62 from 31 non-Hodgkin's lymphomas by PCR assay followed by electrophoresis through 40 cm long, 1.2% agarose gels. We also examined lung and lymphoid cancer patients for the presence of microsatellite alterations using three microsatellite markers located at chromosome 3p (D3S1038 -3p25-, D3S1289 -3p21.3-, and D3S1284 -3p13- dinucleotides) and chromosome X (AR -Xq11.2-q12- trinucleotide). PCR assay was performed using radiolabeled nucleotides and amplified products were processed through denaturing acrylamide gels. The frequency of rare alleles in lung cancer patients, 21/96 (22%), was different from that in the control group, 29/171 (14%) ( $P = 0.05$ ), and was 13/62 (21%) in lymphoma patients. Among the rare alleles, a1+4 was the

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Department of Pathology  
Duke University

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# Immune-Based Approaches to Breast Cancer Susceptibility and Treatment


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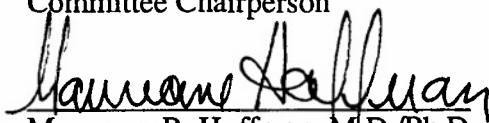
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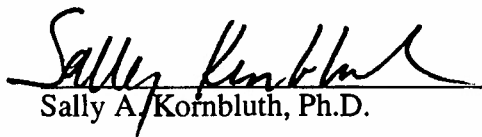
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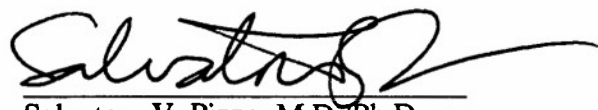
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Dissertation submitted in partial fulfillment of the requirements for the  
degree of Doctor of Philosophy in the Department of Pathology in the  
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1998

Abstract  
(Pathology)

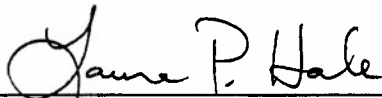
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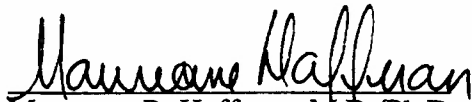
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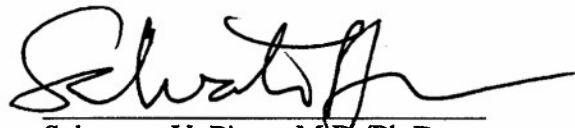
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An abstract of a dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the Department of  
Pathology in the Graduate School of Duke University  
1998



# Abstract

Breast cancer is one of the leading causes of cancer deaths for women in the United States. Women who inherit a mutated form of the BRCA2 gene have a very high probability of developing breast cancer. Very little is known to date about the protein product of this gene or its normal biological function. We have developed immunological reagents against the BRCA2 protein in order to study some of these questions. Using these reagents, we have shown that this protein is expressed at high levels in several epithelial tissues, suggesting a potential role for BRCA2 in processes of cellular differentiation. Further elucidation of the role BRCA2 plays in normal breast biology may help us to understand why tumorigenesis occurs in hereditary cases, as well as non-inherited breast cancer.

Prevention of metastatic breast cancer is limited to early detection and surgical resection of a localized tumor. Treatment for advanced stages of breast cancer is often unsuccessful due to micrometastasis of the tumor prior to therapy. Thus, new methods to treat and prevent metastatic breast cancer are sorely needed. Immunotherapy, which utilizes the specificity of the immune system to target the destruction of tumor cells, is a very promising form of therapy. Immunotherapeutic approaches are much more specific for cancer cells, and therefore less toxic than conventional therapeutic approaches. In addition, once we have developed a better understanding of how to manipulate the immune system, immunotherapy will potentially be a much more effective approach to cancer treatment, and the use of immune-based "cancer vaccines" may even be used preventatively.

EGFRvIII, a variant form of the epidermal growth factor receptor, is found in a large percentage of breast tumors and thus may be a viable target for immunotherapy. I

have developed a bispecific antibody against both EGFRvIII and the CD3-epsilon T-cell activation antigen to redirect the cytotoxic response of T-cells with a broad range of specificities against breast cancer cells that express EGFRvIII. In addition, I have developed an EGFRvIII-transducing adenovirus which will be useful in active immunotherapeutic protocols targeting the EGFRvIII tumor antigen.

# Acknowledgments

First, I would like to thank my research advisor, Laura P. Hale, MD, PhD, for more than I can put into words here. She is one of the most positive people I know, and this comes across in her enthusiastic outlook on everything in which she is involved, including science. She always managed to put a positive spin on my experimental results, as bleak as they sometimes seemed to me! Her love for science, and her ability to balance her career with other things important in life have been truly inspirational. In addition, Laura possesses rare qualities as an advisor: supportiveness, understanding, open-mindedness, and the ability to discuss rather than inform with her students. Laura has always made time for me when I needed her, and I thank her for the immeasurable amount of time she has contributed to my success.

I would also like to thank the two research technicians in our laboratory, Paula Greer and Jie Li. Paula and Jie have not only contributed significantly to my dissertation work, but they have also been good friends. I can't imagine having found a happier lab environment and co-workers with whom it was truly a pleasure to work for four years. These comments apply as well to Kristina Flores, a fellow graduate student in the lab, whose supportiveness and sweet nature have been invaluable.

My family has been another important source of support. My mother, Anita Gilliam, has been my role model, advisor, and friend, and her unconditional love and support is the most precious thing I have.

In the world of science, I would like to thank the following people: Paula, for teaching me most of the techniques I have used in my thesis work and for a significant contribution to the final product ; Ed Lobenhofer, for providing the BAC1 and 2 constructs and for collaborative work using the 2C9 antibody to characterize BRCA2;

Richard Searce, for the tremendous amount of work he put into the generation of the 2C9 antibody, which has been a fundamental component of my thesis work; and Jie Li for all of her beautiful immunohistochemical studies, another important piece of my project. Thanks to all of our collaborators, in particular Drs. Jeff Marks, Bart Haynes, and Andy Amalfitano, who provided reagents, helped out with specialized techniques, etc.

I would like to thank the members of my committee: Drs. Maureane Hoffman, Sally Kornbluth, Sal Pizzo, and Bart Haynes, for the time they took out of their busy schedules to serve as auxiliary advisors, for their supportiveness, and for their helpful suggestions along the way.

Finally, I would like to thank my friends. The past few weeks have really made me realize what a fantastic, supportive, and loving group of friends I'm blessed to have. Special thanks go to Jenn Quirk, Will Rubin, and Michelle McMurry for their invaluable contributions at the end toward helping me to pull this thing together. (This also includes everyone in my lab who spent the good part of a day helping me to print and paste figures). Also, I'd like to thank Elahe Mostaghel, Sherry Carty, Maria Niswonger, Lorrie Cramer, Karen Steinhauser, Ed Lobenhofer, Mara Casar, and the "book club" women for e-mails, phone calls, flowers, early morning coffee, and for all the other supportive things I'm forgetting to mention. I could never have done this without you all!!!

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## List of Abbreviations

aa	Amino Acid
ABs	Alveolar Buds
ABC	ATP-Binding Cassette
Ad	Adenovirus
APC	Antigen Presenting Cell
AT	Ataxia Telangiectasia
ATCC	American Type Culture Collection
ATM	Ataxia Telangiectasia, Mutated
BCA	Bicinchoninic Acid
BDC	Bile Duct Carcinomas
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
bsAb	Bispecific Antibody
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CK	Cytokeratin
CIP	Calf Intestinal Alkaline Phosphatase
CMV	Cytomegalovirus
CPE	Cytopathic Effects
CS	Cowden syndrome
CTL	Cytotoxic T Lymphocyte
DAB	3,3'-Diaminobenzidine
DMBA	9,10-Dimethyl-1,2-Benzanthracene
DPBS	Dulbecco's Phosphate Buffered Saline

dsDNA	Double Stranded DNA
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbant Assay
ER	Estrogen Receptor
FCS	Fetal Calf Serum
FFPE	Formalin-Fixed Paraffin-Embedded
FITC	Fluorescein Isothiocyanate
FPH	Formamide Prehybridization/ Hybridization
GAP	GTPase-Activating Protein
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GST	Glutathione S-Transferase
HAMA	Human Anti-Mouse Antibodies
HBSS	Hanks Buffered Saline Solution
HeBS	HEPES-Buffered Saline Solution
HFK	Human Foreskin Keratinocyte
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IMDM	Iscove's Modified Dulbecco's Medium
LAK	Lymphokine Activated Killer
LN	Lymph Node
Lob	Lobule type
mAb	Monoclonal Antibody
MAP	Mitogen Activated Protein
MEN2a	Multiple Endocrine Neoplasia type 2a

MHC	Major Histocompatibility Complex
MMS	Methyl Methanesulfonate
MW	Molecular Weight
NMU	N-Nitroso-N-Methylurea
OL	Overlapping
PBLs	Peripheral Blood Lymphocytes
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
P/E	Promoter and Enhancer
PEG	Polyethylene Glycol
PI3-K	Phosphatidylinositol 3-Kinase
PLC- $\gamma$	Phospholipase C-gamma
PMSF	Phenylmethylsulfonyl Fluoride
POL	DNA polymerase
PR	Progesterone Receptor
pTP	Preterminal Protein
PTT	Protein Truncation Test
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAGE	Serial Analysis of Gene Amplification
SCC	Squamous Cell Carcinoma
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulfate
SH2	Src Homology 2
SPORE	Specialized Program of Research Excellence
TAP	Transporter Associated with Antigen Processing
TCR	T Cell Receptor

TDLU	Terminal Duct Lobular Unit
TdT	Terminal Deoxynucleotidyl Transferase
TEBs	Terminal End Buds
TILs	Tumor Infiltrating Lymphocytes
TLCK	N $\alpha$ -p-tosyl-L-Lysine Chloromethyl Ketone
TSA	Tyramide Signal Amplification

# **Chapter 1**

## **Introduction and Background**



# Breast Cancer

## Prevalence and staging of breast cancer:

Breast cancer, the most prevalent cancer in women, affects one out of eight American women and accounts for 30% of new cancer cases a year (Landis, 1998). In addition, this disease is responsible for 16% of all cancer deaths in women, making breast cancer second only to lung cancer as a leading cause of cancer mortality in the female population. Technological advances, such as the mammogram, and increased awareness among the population have played a significant role in the early detection of breast tumors and consequent cure by surgical resection in those patients who are treated early in their disease. However, breast cancer remains a devastating and often times incurable disease for women in whom metastasis or micro-metastasis has already occurred before therapy is instituted. Although most cancers are considered "cured" when a patient has survived for five years following therapy with no evidence of recurrent disease, breast cancer micrometastases may resume growth long after the primary disease has been treated, even up to decades later. This uncertainty of the prognosis gives a diagnosis of breast cancer an element of fear and hopelessness encountered with few other malignancies.

The prognosis of breast cancer patients depends on the stage at which the tumor is diagnosed. Nodal involvement is highly indicative of a poor prognosis, with 10 year survival decreasing from 60%-75% for localized tumors (Stage I disease), depending on the size of the primary tumor ( $T_{1-3}, N_0$ ), to 35% for tumors which have spread to axillary lymph nodes (Stage II disease) with primary tumors of any size ( $N_{1-3}, M_0$ ) (Table 1.1).

### **Manchester Staging System**

Stage I	Breast alone involved $\pm$ overlying skin
Stage II	Breast as for Stage 1 and axillary nodes involved, but mobile
Stage III	Skin invaded, fixed or ulcerated, or tumor fixed to underlying muscle or pectoral fascia
Stage IV	Fixed axillary lymphadenopathy, supraclavicular involvement and/or distant metastases

### **TNM Staging Notation for Breast Cancer**

T <sub>1</sub> *	Tumor less than 2 cm in diameter
T <sub>2</sub> *	Tumor 2-5 cm in diameter
T <sub>3</sub> *	Tumor > 5 cm
T <sub>4</sub>	Tumor of any size with direct extension to chest wall or skin
N <sub>0</sub>	No palpable node involvement
N <sub>1</sub>	Mobile ipsilateral nodes
N <sub>2</sub>	Fixed ipsilateral nodes
N <sub>3</sub>	Supraclavicular or infraclavicular nodes or edema of arm
M <sub>0</sub>	No distant metastases
M <sub>1</sub>	Distant metastases

**Table 1.1: Breast cancer staging.** \*T<sub>1</sub>, T<sub>2</sub>, and T<sub>3</sub> tumors further divide into (a) no fixation and (b) fixation to underlying pectoral fascia or muscle. (Souhami and Tobias, 1995).

### **Epidemiology of breast cancer:**

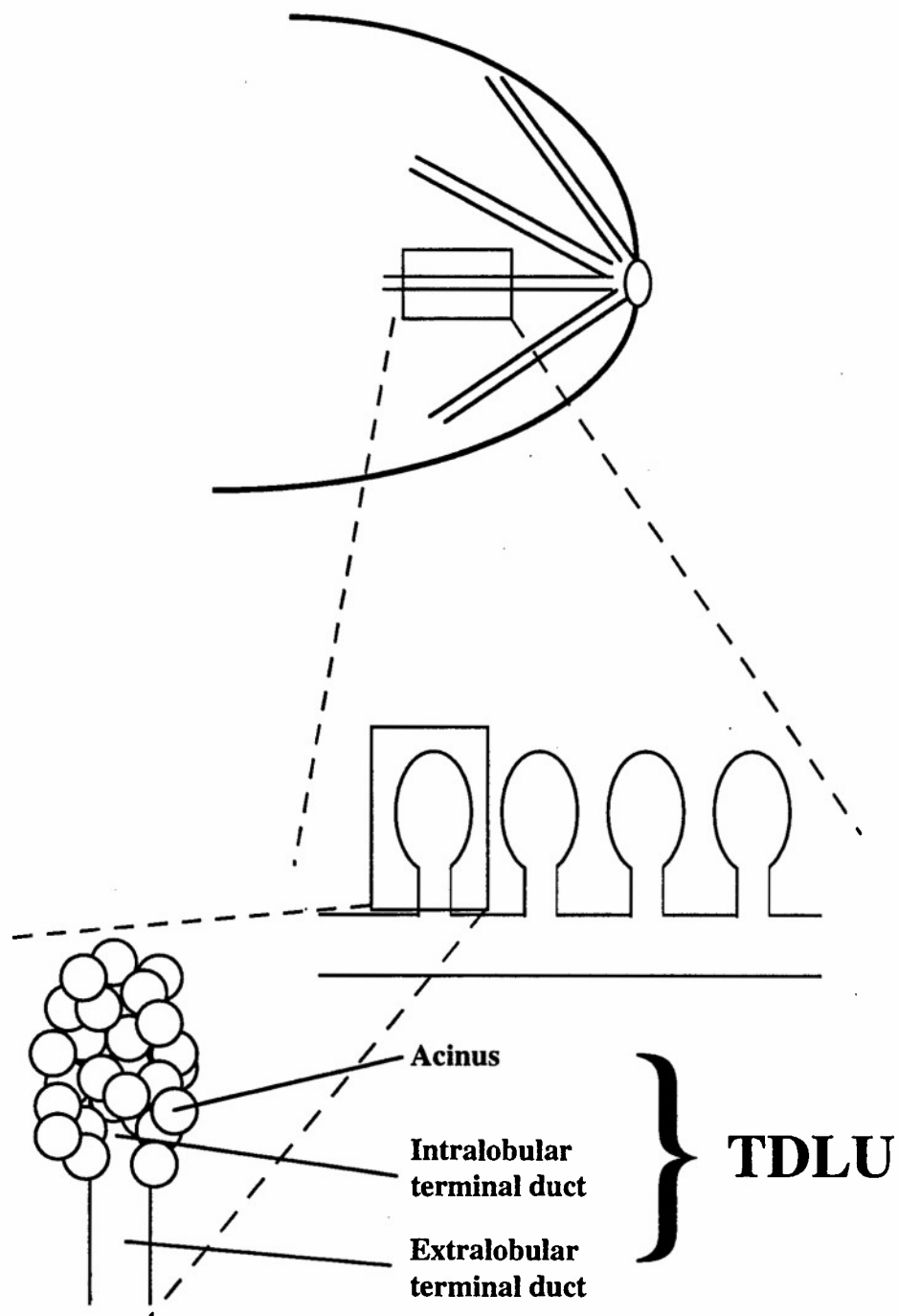
The epidemiology of breast cancer has been extensively studied. The four strongest predictive factors for development of breast cancer are female gender, age, genetic predisposition, and certain aspects of reproductive history (Russo and Russo, 1997). Age and genetic predisposition are not predictors unique to breast cancer. The incidence of most cancers, excluding childhood malignancies, increases with increasing age, due to accumulation of genetic mutations over time. A family history of a first-degree relative with breast cancer diagnosed pre-menopausally increases an individual's risk for developing breast cancer by 3 to 4-fold. Some of the molecular mutations responsible for this increased genetic risk will be described later in this chapter. Risk due to female gender and certain aspects of a woman's reproductive history are not as well understood at the molecular level, but exposure to estrogen appears to be a key element. Aspects of reproductive history that increase the relative risk of developing breast cancer include early age at menarche, late age at menopause, and nulliparity, whereas high parity, long-term breast feeding, and oophorectomy at an early age (<35 years) lower the relative risk. In addition, obesity is associated with a higher relative risk for developing a breast malignancy, likely due to increased estrogen levels produced in peripheral adipose tissue of obese individuals. Thus, increased estrogen exposure appears to be an important contributory factor to development of breast cancer (Hulka, 1997). Interestingly, the age at which a woman has her first pregnancy also seems to be important, with late age (>30 years) at first full term pregnancy associated with increased relative risk for development of breast cancer compared with early age (<20 years) at first full term pregnancy, regardless of the number of pregnancies (and thus the cumulative period of estrogen exposure). This risk factor is probably due to normal changes that occur in the breast with the first pregnancy which will be discussed in more detail later. Some environmental factors have also been identified that appear to confer an increased risk for

developing breast cancer, including alcohol consumption and radiation exposure. Cigarette smoking, although a significant risk factor in many other types of cancers, has actually been found in some studies to be a negative risk factor for the development of breast cancer (Baron, 1990; Palmer and Rosenberg, 1993). A possible explanation for these findings is that cigarette smoking appears to induce a state of relative estrogenic deficiency. As breast cancer is often an estrogen-dependent tumor, the relative risk for the development of breast cancer would therefore be decreased in women who smoke cigarettes (Baron and Haile, 1998). However, the hypothesis that cigarette smoking may reduce the risk for developing breast cancer remains very controversial, and even proponents of this hypothesis are not encouraging the idea of taking up smoking as a means of reducing breast cancer risk.

#### **Normal breast histology and development:**

Despite a solid understanding of the epidemiology of breast cancer, little is known about the mechanisms of initiation or development of this malignancy. An understanding of these processes is crucial for improved prevention and therapy of breast cancer. In order to understand pathogenesis, it is first important to understand the normal biology and development of the breast. Histologically, the mammary epithelium is arranged in 10 to 15 segments, each of which may be likened to a flowering tree (Millis, 1994) (Figure 1.1). The bunches of flowers represent lobules: units composed of individual blind-ending terminal ductules (alternatively called acini), the twigs. The ductules drain into ducts (the branches) and then collecting ducts (the trunk), which open onto the surface of the nipple. The degree of branching of the ductules within a lobule is directly correlated with the degree of differentiation of that lobular unit; similarly, the number of cells per ductule is inversely correlated with stage of differentiation. These different stages of differentiation may be divided into four histologically defined categories (Russo and

Russo, 1997): lobule type 1 (Lob 1) contains clusters of 6 to 10 ductules per lobule. Lobule types 2 and 3 (Lob 2 and Lob 3) are more complex in their branching pattern and have increasing numbers of ductules, with Lob 3 having approximately 80 ductules per lobule. Type 4 (Lob 4), seen during lactation, is the most differentiated structure, with actively secreting acini. The total lobular component in the breast of a post-pubertal, nulliparous woman consists primarily of Lob 1 (50-60%) and Lob 2 (30-35%). However, during a woman's first pregnancy, Lob 1 and Lob 2 structures undergo differentiation into Lob 3 (followed by Lob 4 during lactation). Even after delivery, the breast of a parous woman retains this predominance of the more differentiated epithelium, with Lob 3 structures comprising 80-100% of the total lobular component, until she goes through menopause, at which time a portion of the Lob 3 structures regress to Lob 2 and Lob 1. The more complex histological appearance of the Lob 3 structures is also inversely correlated with proliferative index, as would be expected if these structures are more differentiated. <sup>3</sup>H-thymidine and bromodeoxyuridine (BrdU) uptake studies in addition to studies examining proliferating cell nuclear antigen (PCNA) and Ki-67 staining, which detect cells actively undergoing proliferation (i.e. cells not in G<sub>0</sub>), show that Lob 1 structures have a higher proliferative rate than Lob 2 or Lob 3 structures (Russo and Russo, 1987; Russo and Russo, 1997). Thus, differentiation of the mammary epithelium, which may be quantified by decreased proliferative activity of the epithelial cells and increased complexity of the lobular structure, occurs after puberty and reaches its fully differentiated state only after the first full term pregnancy.



**Figure 1.1: Schematic diagram of normal breast.** The terminal duct lobular unit (TDLU) is composed of several blind-ending units, called acini. These structures drain into ducts, which form an extensive branching network in the breast.

### **Pathogenesis of breast cancer:**

Invasive ductal carcinoma is the most common breast malignancy, representing 76% of breast carcinomas diagnosed (Macdonald and Ford, 1997). Ductal carcinoma is hypothesized to arise from a structure termed the terminal duct lobular unit (TDLU), which corresponds histologically to the type 1 lobule. Type 2 and type 3 lobular structures are also affected by neoplastic processes; however, the invasive behavior of such malignancies decreases with increasing stage of differentiation of the derivative structure (Millis, 1994; Russo and Russo, 1997). For example, lobular carcinoma, a more indolent malignancy characterized by a longer time period between the identification of a primary lesion and the demonstration of invasive behavior, is thought to derive from the more developed Lob 2 structures. This observation suggests that the increased relative risk for malignancy in nulliparous women or women who are older than 30 at the time of their first pregnancy, is directly related to the period of time in which the breast epithelium remains in a relatively undifferentiated state.

This concept that the terminal differentiation induced during the first pregnancy is a crucial factor in the pathogenesis of breast cancer may have even broader implications. For example, inheritance of a mutated form of the BRCA1 gene appears to affect the pattern of differentiation during pregnancy. Examination of breast tissue obtained from prophylactic mastectomies from BRCA1 mutation carriers demonstrates a lack of normal differentiation, even after their first pregnancy (Russo and Russo, 1997). In this case, tissue from parous women is indistinguishable from that obtained from nulliparous women, with a predominance of Lob1 architecture. We have demonstrated that BRCA2, which is hypothesized to function in a similar manner to BRCA1, is expressed in differentiating epithelia. Although a definite function for BRCA2 in differentiation has not yet been demonstrated, BRCA2 may have similar effects on the developmental pattern in the breast tissue of mutation carriers to those described above for BRCA1.

These findings suggest a possible contributory mechanism for the increased risk for developing breast cancer in women who inherit a BRCA1 or BRCA2 mutation. If the time period between puberty and the completion of the first pregnancy represents a "window of high susceptibility" for breast cancer development, then by preventing normal terminal differentiation of TDLUs during pregnancy, the breast tissue of these women remains in a primed state for developing malignancy during their entire post-pubertal lifetime of estrogen exposure, similar to the breast tissue of a nulliparous woman. However, BRCA1 and BRCA2 mutation carriers are at much higher risk for developing breast cancer than are nulliparous women. Thus, this putative contributory mechanism does not fully explain why mutations in these genes are responsible for carcinogenesis. These predisposing genetic factors will be discussed in more detail later.

#### **Animal models of breast cancer pathogenesis:**

Hypotheses about the development of breast cancer in humans are based on snapshot views of the histology of neoplastic and pre-neoplastic lesions found in breast tissue obtained by surgery, autopsy, or occasionally prophylactic mastectomy. Consequently, assumptions must be made about the actual step-by-step occurrence of these processes. Animal models for malignant transformation of mammary epithelium have been invaluable in helping to understand these processes in a context in which the histopathological evolution of malignancy can be directly observed. The Sprague-Dawley rat and the Fischer 344 rat models have often been used for this purpose. These animal models have a very high incidence (nearly 100%) of mammary carcinoma when treated with the specific carcinogens 9,10-dimethyl-1,2-benzanthracene (DMBA) and N-nitroso-N-methylurea (NMU) (Huggins and Yang, 1962; Rogers and Lee, 1986; Russo, 1990). Normal mammary development in these animals is similar to that in humans in many respects; initial development of mammary tissue occurs when the superficial



ectoderm invades the stroma forming structures called terminal end buds (TEBs), which are histologically and developmentally similar to TDLUs in humans. During puberty (day 25-35), these TEBs begin to differentiate into alveolar buds (ABs), which then differentiate into virginal lobules. As with TDLUs in humans, much evidence suggests that the TEB is the structure of origin of mammary carcinogenesis. First, the incidence of tumor development (number of tumors per animal) is directly correlated with the number of TEBs, but not with the number of more differentiated mammary structures. In addition, pregnancy or estrogenic hormonal treatments, which induce differentiation and elimination of the TEBs, make these animals refractory to tumor development. Of note, treatment of these models with carcinogens also induce the formation of benign lesions, but these arise from structures other than the TEBs (such as the more differentiated ABs) and they often appear later than the malignant lesions, suggesting that the benign lesions are not precursors of the malignant lesions, but that two different pathological processes are taking place (Russo and Russo, 1997).

### **Molecular basis of cancer:**

An estimated 10% of breast cancers are genetic in origin, suggesting that 90% of breast carcinomas arise due to a sporadic cause. In the study of both the inherited and sporadic processes involved in cancer development, researchers have seen the birth of a new discipline within the past two decades: the molecular biology of tumorigenesis. This advance has completely changed the way scientists and clinicians view both hereditary and sporadic forms of cancer, and this new understanding has just recently begun to make its way into therapeutics. Cancer genes are divided into two general categories: oncogenes and tumor suppressor genes.

Oncogenes have growth stimulatory functions in cells and lead to tumorigenesis upon activation of latent forms of these genes, termed "proto-oncogenes" via several

mechanisms, including point mutation, amplification, and chromosomal translocation. Because oncogenes act dominantly (one "activated" oncogene is all that is required to induce malignant transformation), they are usually not involved in hereditary forms of cancer. One known exception to this general rule involves a mutated form of the RET oncogene inherited in the Multiple Endocrine Neoplasia type 2a (MEN2a) cancer syndrome (Macdonald and Ford, 1997).

Tumor suppressor genes, on the other hand, normally have growth inhibitory functions or functions in maintenance of genomic stability. Loss or inactivating mutation of both copies of these genes are required for malignant transformation of a cell. For this reason, inherited mutations in these genes are often involved in increased genetic susceptibility to cancer. Inheritance of one mutated allele of a tumor suppressor gene allows for normal embryogenesis and early childhood development due to the presence of the second, normal allele. However, one faulty cell division in which the normal allele is lost is all that is required for tumorigenesis to occur. Hence, familial cancer syndromes have a dominant inheritance pattern, where up to 100% of people inheriting the faulty gene will develop disease, depending on the penetrance of the gene, even though the gene acts recessively at the molecular level.

### **Molecular basis of breast cancer:**

Genetic changes involved in breast cancer have been extensively studied for both sporadic and hereditary forms of this disease. One of the most prognostically significant genetic changes identified to date involves erbB-2, a proto-oncogenic homologue of the epidermal growth factor receptor (EGFR) gene. Changes associated with erbB-2 as well as EGFR are detailed later in this chapter. Another prognostically significant genetic change involves loss of expression of the estrogen receptor (ER) and associated dysregulation of the ER response pathway, often including loss of expression of the

progesterone receptor, as well. ER-negative tumors tend to be more aggressive, are found in younger women, and are associated with shorter disease-free survival periods (Diab, 1996).

Myc is a nuclear transcription factor which, when amplified, is associated with tumorigenesis in a variety of cancers. Myc, complexed with its dimeric partner Max, binds to DNA and presumably controls the transcription of genes involved in cellular proliferation and prevention of differentiation. Dysregulation of these processes by amplification of myc then leads to tumor development due to uncontrolled cellular proliferation or lack of initiation of the differentiation signal. The myc protein has also been shown to induce the apoptotic pathway, although it is not clear how amplification of myc functioning in this capacity would play a role in tumorigenesis (Macdonald and Ford, 1997). Amplification of the myc gene has been found in a subset of breast cancers, with reported incidence varying from 4 to 41% (Bieche and Lidereau, 1995; Macdonald and Ford, 1997). One group has reported amplification in invasive and poorly differentiated carcinomas, with no abnormalities noted in Stage 1 tumors, suggesting that amplification is a later event (Field and Spandidos, 1990). Furthermore, a high incidence of increased levels of myc mRNA and/or protein in tumor tissue is not always correlated with an equivalently high incidence of gene amplification. One study reported that 50% of breast tumors express higher levels of myc mRNA relative to normal tissue, whereas only 11% of these tumors were found to have amplification at the level of the gene, suggesting that myc gene amplification may not be an independent prognostic factor (Field and Spandidos, 1990). Thus, myc's role in the progression of breast tumor initiation and development remains unclear (Macdonald and Ford, 1997).

Ras is another oncogene which has been implicated causally in breast cancer. This gene encodes a molecule with GTPase activity which is involved in activation of signal transduction pathways. Growth stimulatory signals are transmitted from the cell

surface through ras, which either relays the signal via the mitogen activated protein (MAP) kinase pathway to the nucleus, effecting DNA synthesis, or via the alternative phosphatidylinositol 3-kinase (PI3-K), rac/rho pathway, effecting morphological changes in the cell. High levels of ras protein have been reported in a large percentage of malignant breast tumors (63-83%), and increased levels of ras appear to be correlated with larger tumor size and shorter disease-free interval (Field and Spandidos, 1990). At the DNA level, there is little evidence for genetic alterations involving ras; however, there has been evidence suggesting that the inheritance of a certain rare H-ras allele is significantly associated with the development of breast cancer as well as other tumor types, with a 1.7-fold increased risk in heterozygotes, and a 4.6-fold increased risk in homozygotes for developing a malignancy (Krontiris, 1993; Macdonald and Ford, 1997; Weston and Godbold, 1997). This allelic variant may therefore potentially contribute to the incidence of hereditary breast carcinoma, although this remains to be confirmed.

#### **Molecular basis of hereditary breast cancer:**

Several genes have been identified, including p53, ATM, BRCA1, and BRCA2, in which a familial inheritance pattern has been clearly defined for breast cancer development. Evidence for a familial inheritance pattern includes clustering in families, early age of onset, bilateral breast tumor development, and increased incidence of other types of tumors within a family.

BRCA1 and BRCA2, which will be discussed later in more detail, are considered the classical "breast cancer susceptibility genes" and account for an estimated 80% of familial breast cancers.

P53 is a tumor suppressor gene involved in checkpoint control of the cell cycle and apoptosis. Inheritance of a mutated copy of this gene results in the Li-Fraumeni syndrome, an autosomal dominant disorder conferring a 25-fold increased risk for the

development of a wide range of malignancies (Evans and Lozano, 1997). P53 mutations are also associated with a high percentage of sporadic breast carcinomas. One recent point of controversy has been the prognostic significance of p53 mutations in breast tumors. Accumulated evidence seems to suggest that the detection of mutant forms of p53 in breast tumors is strongly indicative of poorer prognosis; however, a causal relationship between this genetic alteration and breast cancer initiation and/or progression has yet to be clearly elucidated (Macdonald and Ford, 1997).

The genetic basis of Ataxia Telangiectasia (AT), an autosomal recessive disease characterized by cerebellar degeneration, chromosomal abnormalities, immunological defects, development of multiple ocular telangiectases (dilated blood vessels), and cancer predisposition, was recently found to be due to mutation of the ATM (ataxia telangiectasia, mutated) gene. This gene encodes a large protein with a PI3-K-like domain and homology to yeast proteins involved in cell cycle control, cellular responses to DNA damage, and meiotic recombination (Savitsky, 1995). ATM-null cells are sensitive to ionizing radiation, further supporting the hypothesis that this gene product is important in DNA repair processes (reviewed in Brown and Tagle, 1997). Early studies of AT families indicated that female relatives of AT patients presumed to be heterozygous for mutated ATM do not develop AT but are at increased risk for developing early onset breast cancer (Macdonald and Ford, 1997). However, two recent studies analyzing germ-line mutations of the ATM gene in a population of women with early onset breast cancer suggested that the contribution to early onset breast cancer conferred by heterozygous ATM mutations was minimal (FitzGerald, 1997; Chen, 1998a). A third study suggested that an increased risk for breast cancer was associated with the inheritance of heterozygous ATM mutations conferring less severe clinical phenotypes, often caused by point mutations rather than truncating mutations (Stankovic, 1998). These mutations would therefore have been missed in the previous two studies

which identified mutations by the protein truncation test (PTT). Thus, the contribution of mutations in the ATM gene to hereditary breast cancer remains controversial.

Another inherited cancer syndrome in which the molecular basis has recently been elucidated is Cowden syndrome (CS). This autosomal dominant disorder characterized by multi-organ hamartoma development and predisposition to certain cancers, including breast cancer, is associated with mutations in the PTEN gene (also called MMAC-1). The PTEN gene encodes a protein tyrosine phosphatase homologous to tenascin (reviewed in Eng, 1998). Although population-based screening studies have indicated that only a small fraction of primary breast cancers are due to mutations in PTEN, these studies support the hypothesis that PTEN mutations are associated with breast cancer in CS patients (Lynch, 1997; Rhei, 1997).

With the continued study of chromosomal abnormalities found in inherited breast cancers, and with technological advances such as serial analysis of gene amplification (SAGE) making possible the rapid screening of breast cancer cDNA libraries for tumor-specific genetic alterations (Velculescu, 1995; Zhang, 1997), our knowledge regarding the genes involved in and/or responsible for the development of breast carcinoma will continue to expand.

### **Therapy for Breast Cancer:**

Surgery and radiation are the primary modes of therapy for women diagnosed with breast cancer. For local disease, the modified radical mastectomy has been largely replaced by lumpectomy, followed by post-operative radiotherapy to control local recurrence. Use of hormone antagonists such as the anti-estrogenic agent tamoxifen may also be prescribed. Such hormonal therapy represents the most significant recent advancement currently in use in breast cancer treatment, as it is relatively non-toxic and has a very high response rate (70%) in women with estrogen and progesterone receptor

positive (ER+/PR+) tumors. Unfortunately, only 65% of cancers in postmenopausal women are ER+/PR+, with an even smaller fraction of tumors positive in premenopausal women (35%), and previously responsive tumors often become resistant to hormonal therapy over time (Souhami and Tobias, 1995). For women with metastatic disease, high dose chemotherapy and bone marrow transplantation is currently the only potentially curative treatment.

Chemotherapy, like radiation therapy, is toxic for dividing cells in the body; hence, its relative specificity for tumor cells lies in the fact that these cells are rapidly proliferating. However, chemotherapy is also toxic to normal dividing cells, which accounts for many of its serious and potentially life-threatening side effects, such as bone marrow suppression and gastrointestinal mucositis. Furthermore, although chemotherapy has a very high cure rate for some types of cancers including acute lymphoblastic leukemia, gestational trophoblastic disease, and testicular cancer, its usefulness has been limited in treating breast cancer. Therefore, new types of therapy to prevent or cure metastatic disease are sorely needed. One such novel treatment option is immunotherapy, a specific and relatively non-toxic form of therapy which is just now being tested in certain clinical settings.

## BRCA2

Individuals with germline mutations in the BRCA2 breast cancer susceptibility gene are at increased risk for breast cancer (including male breast cancer), ovarian cancer, pancreatic cancer, prostate cancer, and potentially other malignancies (reviewed in Stratton, 1996). The BRCA2 gene was localized in 1994 and identified by positional cloning in 1995 (Wooster, 1994; Wooster, 1995; Tavtigian, 1996). The predicted amino acid sequence of BRCA2 shows no significant homologies to other known proteins, including the first identified breast cancer susceptibility gene, BRCA1. However, BRCA2 shares some structural features with BRCA1, as well as similarities in expression pattern and hypothesized functions. Thus, advances made in understanding the function of the BRCA1 protein product may also have implications for BRCA2. More is known about BRCA1, as this gene was identified a year earlier than BRCA2; therefore, references to BRCA1 will be included in this discussion where relevant to BRCA2.

The BRCA2 gene consists of 27 exons extending over 70 kB, and produces a cDNA of 11,385 bp encoding a 3418 aa protein with a predicted MW of 384 kDa (Figure 1.2). Structural features of the predicted BRCA2 protein include a highly charged amino acid composition, a putative nuclear localization sequence (McAllister, 1997), a C-terminal domain with possible grainin homology (Jensen, 1996) of disputed significance (Mazoyer, 1996), and absence of an obvious transmembrane sequence or an N-terminal signal sequence for secretion (Tavtigian, 1996). BRCA1 is also a very large (220 kDa), highly charged protein with a putative grainin domain (Jensen, 1996) and nuclear localization sequence (Wilson, 1997). In addition, both BRCA1 and BRCA2 contain a very large exon 11 (approximately 5 kB for BRCA2). Although the BRCA2 gene is

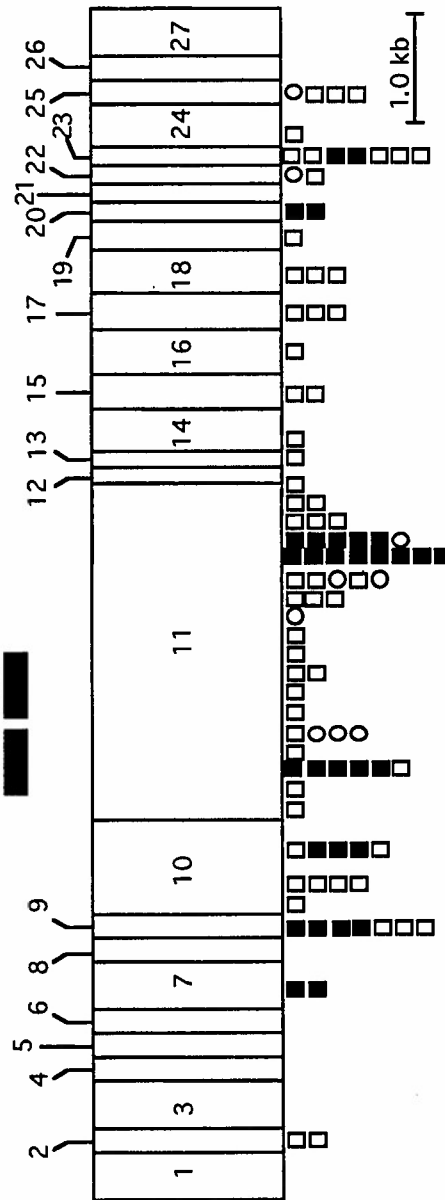
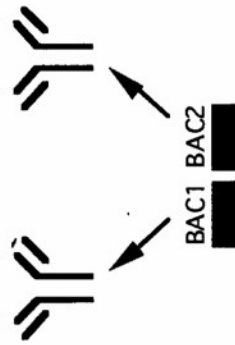


**Figure 1.2: BRCA2 structure, mutations, and identification of protein regions used to produce antibodies.** The BRCA2 gene is composed of 27 exons (numbered in the figure), with a cDNA of 11,385 bp. A representative subset of mutations which have been described for BRCA2 is shown in this figure. Mutations have been found throughout the coding sequence, including frameshift (darkened square), non-sense (open square), and missense (circle) mutations. Monoclonal and polyclonal antibodies against BRCA2 have been generated in our laboratory (refer to Chapter 2). The protein products of two non-overlapping regions of BRCA2 exon 11 were used as immunogens for the generation of these immunological reagents, as shown in this diagram. Figure adapted from (Kahn, 1996).

mAb:  
2C9

Mouse Polysera:  
M#1, M#2, M#3

Mouse Polysera:  
M#4, M#5, M#6



generally poorly conserved between species (human BRCA2 is 58% and 59% homologous to rat and mouse, respectively (McAllister, 1997), Bork *et al.* described 8 copies of a 30-80 aa internally repeated domain within exon 11 (BRC repeats), four of which are highly conserved between rat, mouse, and human (Bork, 1996; Bignell, 1997) and appear to play an important role in BRCA2 function (see below). Aside from these structural clues, sequence analysis of the BRCA2 gene and its predicted protein product has not contributed significantly to a better understanding of either the normal cellular functions of this protein or how mutation of the gene contributes to development of breast cancer.

The BRCA2 gene is widely transcribed but at relatively low levels in most tissues (Wooster, 1995; Tavtigian, 1996). Initial tissue screens demonstrated moderate expression of BRCA2 mRNA in normal breast tissue (Tavtigian, 1996), with high levels found in thymus and testis, two organs characterized by normally high levels of proliferation and differentiation. High levels of BRCA2 mRNA were seen in murine breast only during development of terminal end buds during puberty and in differentiating alveoli during pregnancy (Rajan, 1996). Other organs with significant BRCA2 mRNA expression include ovary, spleen, eye, and certain areas of the brain (Connor, 1997a). BRCA2 mRNA expression is regulated by the cell cycle in breast and ovarian epithelial cell lines, with increased BRCA2 mRNA detected in late G1 and early S phases (Rajan, 1996; Vaughn, 1996). The kinetics as well as the tissue distribution of BRCA2 mRNA expression is similar to that observed for BRCA1, leading to the suggestion that these two proteins are regulated in a coordinate fashion (Rajan, 1996; Vaughn, 1996). BRCA2 mRNA has been reported to be upregulated in cultured mammary epithelial cells in response to estrogen (Spillman and Bowcock, 1996), as well as in differentiating mammary epithelial cells in response to glucocorticoids (Rajan, 1996).

Mice genetically homozygous for BRCA2 truncated prior to exon 11 ("BRCA2-negative") show growth retardation beginning at embryonic day 6.5 and die by embryonic day 8.5 (Ludwig, 1997; Suzuki, 1997; Sharan, 1997b). Post-mortem histologic examination demonstrates lack of appropriate cell numbers and decreased proportion of proliferating cells, leading to the hypothesis that BRCA2 is important in cell proliferation. BRCA2-negative embryos have decreased levels of cyclin E, decreased levels of mdm2, and increased levels of p21 suggesting that BRCA2 protein interacts with the p53 pathway and may similarly function as a "guardian of the genome", to protect the integrity of DNA replication (Suzuki, 1997). BRCA2-negative embryonic stem cells are extraordinarily sensitive to radiation exposure which results in double stranded (ds) DNA breaks (Sharan, 1997b).

More recent studies have demonstrated that viable mice can be generated using BRCA2 truncated at bp 6038, beyond the BRC repeat regions in exon 11 (Connor, 1997b). These mice exhibit growth retardation and die by week 22, primarily of thymic lymphoma. Levels of p21 and p53 are consistently elevated in embryonic fibroblasts homozygous for the truncated BRCA2, however induction of p53 by ionizing radiation appears intact, suggesting that BRCA2 is not involved in sensing dsDNA breaks upstream of p53. Cells homozygous for truncated BRCA2 require increased time to repair dsDNA breaks generated by ionizing radiation, suggesting that BRCA2 is necessary for efficient repair of dsDNA breaks. Studies using the yeast two-hybrid system report that, similar to BRCA1, BRCA2 protein interacts with Rad51, a component of the synaptonemal complexes important in DNA exchange processes during meiosis, and a homologue of the bacterial RecA protein (Shinohara, 1992; Mizuta, 1997; Scully, 1997; Sharan, 1997b). Furthermore, a recent report has demonstrated that Rad51 binds BRCA2 via the BRC repeat regions, and these domains are required for resistance to methyl methanesulfonate (MMS), which putatively induces double-stranded DNA

breaks, in cells lacking full-length BRCA2 (Chen, 1998b). Taken together, these studies have led to the recent proposal of a "caretaker" role for BRCA2 protein in monitoring and/or repair of DNA double-strand breaks (Kinzler and Vogelstein, 1997; Sharan, 1997b).

Recently, BRCA2 protein has been immunoprecipitated from nuclear fractions of breast and bladder carcinoma cells, and its expression was shown to be upregulated with the cell cycle, with induction at late G<sub>1</sub>/early S phase (Bertwistle, 1997; Chen, 1998b). However, no descriptions of the normal cellular and tissue distribution of this protein have been published to date.

## **EGFRvIII**

The wild-type epidermal growth factor receptor (EGFR) is a 170kDa transmembrane protein encoded by the 110 kb EGFR gene located on the short arm of human chromosome 7. This protein serves to transmit growth stimulatory signals from the surface of the cell to the cell's interior. The signal transduction properties of EGFR derive from its activity as a protein tyrosine kinase. Binding of EGF to the EGFR on the cell surface induces dimerization of two receptors, which then cross-phosphorylate each other on tyrosine residues of the cytoplasmic domains of these proteins. The phosphotyrosines then serve as binding sites for the SH2 domains of other important signaling molecules, including phospholipase C-gamma (PLC- $\gamma$ ), PI3-K, and GTPase-activating protein (GAP) (reviewed in Cooper, 1995). Because of EGFR's role in the proliferative signaling pathway, it is not surprising that overexpression of the structurally normal EGFR tyrosine kinase is sufficient to induce transformation. Amplification of EGFR has been reported in a number of human neoplasms, including breast carcinoma (Ro, 1988), gliomas (Libermann, 1985), bladder carcinoma (Berger, 1987) squamous

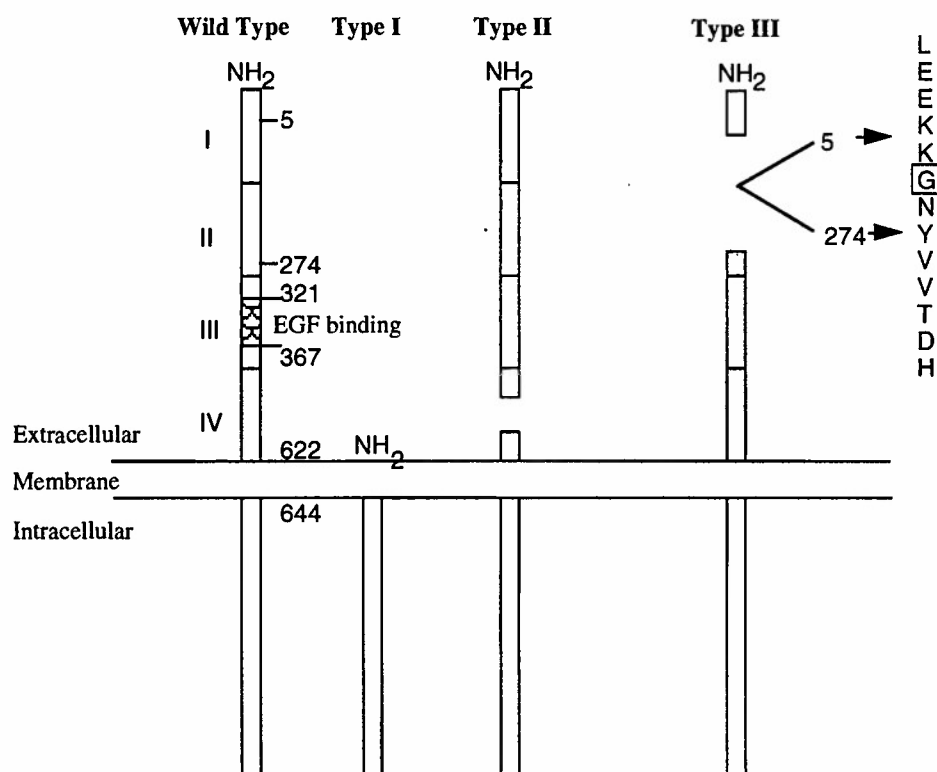
carcinoma cell lines (Yamamoto, 1986), and an epidermoid carcinoma cell line (A431) (Ullrich, 1984). In breast cancer, amplification of EGFR has been reported in 20% of primary tumors, and this finding is strongly associated with early recurrence and death in lymph node (LN)-positive patients (Horak, 1991). Likewise, in biopsy tissue of gliomas in which the EGFR gene was amplified, it was demonstrated that these cells expressed extrachromosomal double minutes which contain the amplified genes (Bigner, 1987).

In addition to gene amplification, cellular tyrosine kinase receptors may be activated for neoplastic transformation via specific molecular events, such as point mutations or deletions. ErbB-2 (also known as HER-2/neu) is a proto-oncogene encoded on human chromosome 17 that represents a truncated version of the wild-type EGFR from which the EGF-binding region has been deleted. The consequence of this deletion is to produce a constitutively active protein-tyrosine kinase that is no longer dependent on EGF binding to the receptor for transmission of the growth-stimulatory signal. Gene transfer experiments have demonstrated that high-level expression of normal erbB-2 is sufficient to induce cell transformation, and amplification of erbB-2 is particularly common in human breast and ovarian cancers, having been detected in approximately 25% of these neoplasms (Slamon, 1989). Furthermore, the amplification of this oncogene product has been associated with a worse clinical prognosis. Patients with high-level expression of erbB-2 have more aggressive tumors and historically lower survival rates (Guerin, 1988; Tsuda, 1989; Tsuda, 1990; Schroeter, 1992).

As previously noted, the EGFR gene is often amplified and overexpressed in particular types of neoplasms, and frequently this amplification is correlated with structural rearrangement of the gene, resulting in the expression of a subset of unique tumor-specific antigens. This observation dates back to 1985, when Libermann et al. first reported the amplification, enhanced expression, and possible rearrangement of the EGFR gene in 4/10 glioblastoma multiforme patient biopsies (Libermann, 1985). Since that

time, Dr. Darell Bigner at this institution as well as others have demonstrated that rearranged variants of the EGFR are expressed in a variety of malignancies, including glial malignancies (Humphrey, 1988; Bigner, 1990; Ekstrand, 1992; Wong, 1992), non-small cell lung carcinomas (Garcia de Palazzo, 1993), squamous cell carcinomas of the head and neck (Hunts, 1985), and breast carcinomas (Wikstrand, 1995). In gliomas, the presence of these variant EGFRs correlates with high tumor grade (e.g. they are found in glioblastoma multiforme) and poor prognosis (Lund-Johansen, 1990). As the amplified EGFR genes are found in unstable double minute chromosomes in these malignant gliomas, expression of variant forms of EGFR is lost over time with *in vitro* culture. Therefore, human gliomas known to express these variant forms of EGFR have been propagated as xenografts in immunodeficient mice (Bigner, 1990).

Using this system, Bigner's laboratory has characterized a subset of EGFR variants found in human gliomas and reported three distinct rearrangements, denoted type I, type II, and type III (Wong, 1992) (Figure 1.3). All three rearrangements result in in-frame deletion-mutant extracellular domains of the wild type EGFR protein. The most frequently detected variant, type III (denoted EGFRvIII), is characterized by an 801 base pair deletion resulting in the loss of 267 amino acids spanning the first and second extracellular domains of the receptor, with generation of a glycine residue at the novel splice site. EGFRvIII binds EGF, but with markedly decreased affinity relative to structurally normal EGFR. Furthermore, the transforming potential of EGFRvIII is ligand-independent and unregulated *in vivo* and *in vitro* (Batra, 1995). Both of these observations suggest that naturally occurring EGFRvIII, like erbB-2, may play an oncogenic role in the tumor cells in which it is expressed, since these abnormal proteins may be capable of functioning in the absence of EGF. Consequently, they would have a growth advantage over cells expressing the structurally normal EGFR in the presence of low levels of EGF (Bigner, 1990).



**Figure 1.3: EGFR and variant forms of EGFR.** Expression of variant forms of the epidermal growth factor receptor has been described in several malignancies. Deletions of extracellular regions are seen in each of these variant EGFR proteins. EGFRvIII is expressed at high levels in gliomas and at lower levels in other tumors, including breast cancer (see Chapter 4). In this variant EGFR, a 267 amino acid deletion occurs at the N-terminal end of the protein, resulting in a truncated extracellular domain and a novel, immunologically detectable region at the fusion junction, as indicated. The EGF binding region, as highlighted on the wild type EGFR, is present in types II and III variants, although biological responses to EGF are altered in these variants.



The most significant finding with respect to EGFRvIII, however, is that this protein appears to be primarily expressed in particular types of malignancies in adult tissues. To study the expression pattern of this protein, our lab used monoclonal antibodies against EGFRvIII which were generated by immunization with a 14-amino acid peptide (Pep-3) corresponding to the predicted amino acid sequence at the fusion junction (Figure 1.3), followed by immunization with EGFRvIII expressing cells or cell membranes (Humphrey, 1990; Wikstrand, 1995). Using these monoclonal antibodies, EGFRvIII expression was examined immunohistochemically in a number of tissues. In human gliomas, expression of EGFRvIII was detected in 52% of the tissues examined - a higher incidence than was previously reported by studies that investigated EGFRvIII expression using polyvalent antisera (Wikstrand, 1995). These studies further demonstrate that this antigen is expressed at high levels in the gliomas that stain positively for EGFRvIII (Figure 1.4). We have also demonstrated the presence of EGFRvIII protein in high percentage of breast carcinoma tissues, using immunohistochemical analysis, RT-PCR, and flow cytometry (refer to data in Chapter 4). Until recently, no normal adult tissues tested, including those from the peripheral and central nervous system, skin, breast, liver, lung, ovary, testes, kidney, and colon have been found to express EGFRvIII by immunohistochemical and/or genetic analysis (Wikstrand, 1995). Although we have recently demonstrated low level reactivity with anti-EGFRvIII mAb L8A4 by normal human thymocytes, this protein is expressed at much higher levels in tumor tissues. Thus, EGFRvIII is a tumor-associated antigen expressed widely in tumor tissues and may be a useful target for specific therapies including immunotherapy.



**Figure 1.4: Demonstration of EGFRvIII in routinely processed patient tumor tissue.** Formalin-fixed paraffin-embedded sections of a human glioblastoma were reacted with either control antibody IgG1 (top panel) or EGFR-specific mAb, L8A4 (bottom panel), after microwave citrate antigen retrieval (see Materials and Methods). High level positive reactivity around the cell borders (indicated by dark brown color) after incubation with L8A4 but not with the control antibody indicates that this tissue strongly expresses EGFRvIII.

# Immunotherapy

The idea that host defenses (i.e. the immune system) provide protection against the overgrowth of tumor cells that arise continuously in the body dates back to the early 20th century but did not become important until the late 1950's and the 1960's. During these decades, researchers began to view the cellular immune system as an evolutionary adaptation to defend against tumor cells, as no other function for the cellular immune system, other than allograft rejection, was understood at that time. Macfarlane Burnet was one of the early proponents of an extended hypothesis of immunological resistance against tumor cells, and he coined the term "immunosurveillance" to describe the hypothesis that "an important and possibly primary function of immunological mechanisms is to eliminate cells which as a result of somatic mutation or some other inheritable change represent potential dangers to life" (Burnet, 1970).

It is clear that there is an interaction between tumors and the immune system, but the importance of this interaction in defense against cancer remains controversial. Proponents of the concept of immunosurveillance believe that this "policing" activity of the immune system plays a very important role in the day-to-day prevention of the development of cancer in an individual. Observations cited by Burnet to support the important role of immunosurveillance include the following: 1) the development of tumors is more likely to occur at stages in life when the immune system is in some way compromised (early in life or in old age); 2) persons with genetically inherited immunological deficiencies are more likely to develop neoplasms than are individuals with normal immune systems; 3) immunosuppressive agents (chemotherapeutic agents as well as radiation therapy) increase the likelihood of developing tumors; 4) autopsy examination of tissues from clinically normal individuals demonstrates a much higher

incidence of histological malignancy than would be predicted from epidemiological data, suggesting that many of these neoplastic foci would *not* have evolved into clinical cancers had the person survived; 5) occasional spontaneous cures of cancer occur, even when the therapeutic measures would not be expected to eliminate all the neoplastic cells; and 6) an active lymphocytic infiltration into the tumor site after surgical removal is associated with an improved survival for these patients (Burnet, 1970).

In addition to seeking answers by clinical observation to the question of whether the immune system provides protective immunity against tumor cells, the concept of immunosurveillance has been under experimental investigation for many years. One of the earliest advances in this area came from studies of tumor transplantation in mice during the 1940's-1960's. Once inbred strains of mice had been produced so that transplants could be accomplished without rejection due to histocompatibility differences, the transplantation of chemically induced cancers in syngeneic strains could be studied. Significantly, these studies demonstrated that a recipient mouse could be immunized against tumor transplants from a syngeneic animal, suggesting that the cancer cells must express an altered antigen (denoted a "tumor-specific rejection antigen") that was recognizable as foreign by the recipient mouse (Gross, 1943).

These tumor-specific rejection antigens were further defined with the development of cloned cytotoxic T lymphocyte (CTL) lines which were shown to have highly specific activity against particular tumor antigens. *In vitro* studies with these CTL clones allowed researchers to better define which epitopes of tumor antigens played an important role in the immune system's ability to recognize and reject the tumor. By culturing the parental tumor with a specific CTL clone, Wortzel, et al. were able to select for "antigen-loss" variants (Wortzel, 1985). When reinjected into the same strain of mouse from which the parental tumor originated, the investigators found that the loss of particular antigens converted a benign tumor into a malignant phenotype. This suggested

that the epitopes which had been lost during the selection process were normally important for recognition of this particular type of tumor by the immune system of these mice. Thus, enhancing the body's own natural response, generating a novel immune response, or utilizing the exquisite specificity of components of the immune system against these tumor specific antigens has been the thrust of many investigators' efforts to improve therapy for metastatic disease.

The choice of useful targets for immunotherapy requires a clear understanding of the cellular and immunologic mechanisms underlying the generation of an anti-tumor response. The anti-tumor response may be divided into two phases. In the induction phase, antigens released from degraded tumor cells are phagocytosed by professional antigen presenting cells (APCs), which go to draining lymph nodes and present antigen to naive T cells. In the expansion phase, the newly activated tumor-specific T cells emigrate from lymph nodes to the periphery where they encounter tumor cells. This second encounter with the specific antigen on the tumor cells provides the signal which directs the T-cell either to proliferate further, to act as an effector by secreting cytokines, or to kill the tumor cells.

Anti-tumor immunotherapy may also be categorized according to whether or not immunological memory is induced, which generally corresponds to the branch of the immune system involved. With active immunotherapy, an anti-tumor immunological response is generated in the host, resulting in the production of memory cells and an anamnestic response if the target antigen is encountered at a later time. This type of immunotherapy is usually mediated by the cellular immune system, primarily by CD8+ cytotoxic cells. Passive immunotherapy involves the use of components of the immune system, such as antibodies, to target tumor cells without actually generating an immune response in the host.

The induction of immunological memory, resulting in long-term protection from tumors expressing antigens against which the individual has been immunized, makes active immunotherapy optimal. However, effective anti-tumor immune responses have proven very difficult to generate for several reasons. As with vaccination against foreign antigens, there are very strict requirements for the generation of an immune response; consequently, certain peptides are simply non-immunogenic in a subset of the population. These strict requirements for generating immunity are first dependent on the APC's ability to present an antigen. Individual variation in the proteosomes (enzymes that proteolytically cleave a protein into short peptides), transporters associated with antigen processing (TAP-1 or TAP-2, which transport processed peptides from the cytosol into the endoplasmic reticulum for association with major histocompatibility complex proteins), or chaperones (which shuttle intermediates) may determine which particular peptide is presented by an individual.

Next, generation of an immune response requires that the antigen be presented in the context of major histocompatibility complex (MHC) proteins, a process in which the *particular* processed peptides *selectively* interact with particular polymorphic MHC molecules. Thus, having a certain HLA (human leukocyte antigen) haplotype may determine whether or not an individual will generate an immune response against an antigen. Finally, the T cell receptor (TCR) on an antigen-specific, primed T cell must interact with the processed antigen-class I MHC on an appropriate target cell, leading to the formation of a T-cell/target-cell conjugate. Formation of this complex results in signal transduction through the CD3 complex, activating the T cell and ultimately resulting in the response - either lysis of cells expressing the target antigen by cytotoxic T-cells, or IL-2 release by helper T-cells. The density of the peptide-MHC complex on the surface of the cell is also very important, as the intensity of the immune response increases with increasing antigen density (Karjalainen, 1994). Furthermore, there is a

great deal of interplay between the different branches of the immune response. At the time of the initial T-cell/APC interaction, differentiation of a CTL precursor into an activated CTL requires that a second signal be provided, such as IL-2 release by activated CD4<sup>+</sup> cells. Clearly, generation of an immune response is a complex process, and this makes an understanding of how to manipulate the immune system to respond crucial for successful active tumor vaccination approaches.

In addition to the complications mentioned in the previous paragraph, the generation of an anti-*tumor* immune response poses an even greater challenge for two reasons. First, few antigens produced by tumor cells are truly non-self antigens, with the exception of mutated oncogenes or tumor suppressor genes which are only present in the tumor cells themselves. Most tumor antigens are proteins recruited by the tumor cell as a means of enhancing proliferative capacity, metastasis, or other important tumorigenic functions. These proteins may not be expressed in normal adult tissues but are often expressed during developmental periods (oncofetal antigens) or are expressed during wound healing, regrowth of a tissue following tissue damage, etc. Consequently, all potentially responsive TCRs may have been clonally deleted during thymic development because of the manner in which the antigen was first presented to the immune system. Second, tumors have evolved very sneaky mechanisms of evading destruction by the immune system. For example, immune destruction of malignant cells is limited to those tumors which express MHC class I antigens. Tumor cells commonly downregulate class I MHC, preventing the recognition and destruction of the tumor cells by the immune system (Garrido, 1993). This defense would also be effective against vaccine-generated immune responses and might even be selected for by such a therapy. In addition, the effective generation of an immune response requires that a second signal be given, in addition to the TCR/MHC + peptide recognition signal. These signals include cytokine/cytokine receptor interaction and ligation of CD28 on the target cell with B7 on

the effector cell. The absence of a second signal provided by tumor cells is one of the main problems encountered in tumor immunotherapy, and tactics for bypassing this "evasion strategy" are currently being explored. Clearly, generation of a cytotoxic anti-tumor immune response is a complex process, and an alternative means of utilizing the specificity of the immune system while bypassing its restrictions would be extremely useful for cancer therapy.

In light of the aforementioned complications, several different immunotherapeutic approaches to treating tumors have been proposed and/or tested. One approach involves administration of a patient's own lymphocytes which have been activated *in vitro* (lymphokine activated killer, or LAK cells) as adjuvant therapy for metastatic or micrometastatic disease (reviewed in Sussman, 1994). By activating cells *in vitro*, the requirement for appropriate activation signals *in vivo*, which are often absent in the case of tumor immunology, is bypassed. This approach was initially tested for melanomas as well as other malignancies, with mixed results (Reintgen, 1991). Drawbacks include limitation of the patient's immune response by the number of lymphocytes that can be removed, treated, and reinfused, and the lack of generation of immunological memory. Furthermore, the treatment is patient-specific and costly.

Another immunotherapeutic approach involves transfection of cytokines into the tumor cells themselves to form an autologous tumor vaccine. The cytokines stimulate development of an immune response against antigens present on the tumor, which is also active against unmodified tumor cells. This approach is thus another means of "exposing" the non-immunostimulatory tumor antigens to the immune system by providing the second signal required to induce a response. These protocols have shown moderate success in animal studies (Gilboa, 1994; Sampson, 1996) and clinical trials using this approach in melanoma, breast, and prostate cancer are beginning at this institution. Drawbacks include the requirement for fresh, sterile tumor that can be



cultured during the time in which the transfection is performed. In addition, ethical issues are involved in the readministration of tumor cells to the patient. Finally, as with the *in vitro* activation of lymphocytes, each treatment is patient-specific and therefore costly.

A third immunotherapeutic approach under investigation is the administration of tumor-specific antibodies conjugated to a toxin or radionuclide to enhance the specificity of an otherwise non-specific chemotherapeutic agent or radiation therapy (reviewed in (Pai and Pastan, 1994)). Such an approach is being studied at this institution in phase I and phase II trials with promising results (Bigner, 1998). Disadvantages to this approach include the lack of generation of immunological memory, the possibility of hypersensitivity reactions against the antibody used if the murine antibodies used are not chimerized or humanized, down-regulation by the tumor of the antigen against which the antibody has specificity, and/or resistance of the tumor cells to killing despite antibody binding. In addition, non-specific tissue trapping of the anti-tumor antibody poses problems for specific tumor targeting using this therapy.

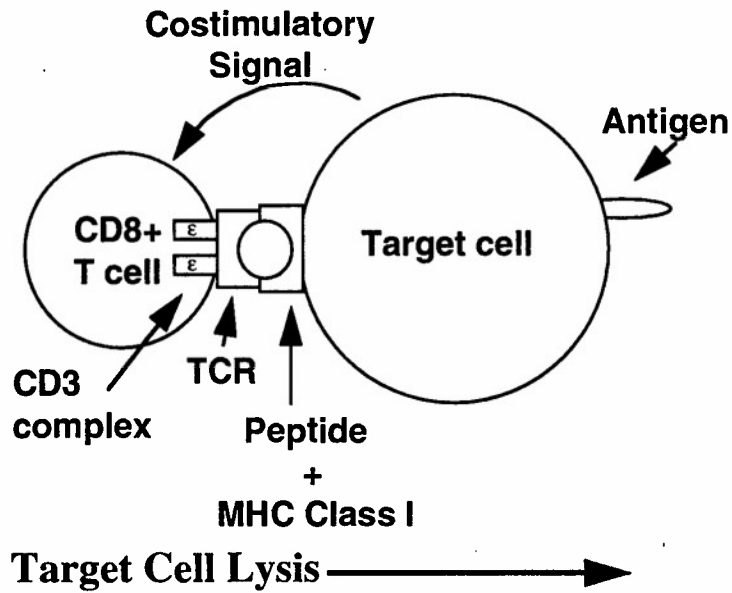
The generation of bispecific antibodies is a variant on the approach which uses monoclonal antibodies to target anti-tumor therapy. Bispecific antibodies utilize the specificity of the humoral immune system to redirect the cellular immune system against tumor cells bearing an antigen against which the antibody is directed (Figure 1.5). A great deal of evidence has accumulated suggesting that the use of bispecific antibodies or other bispecific constructs will be useful as a means of targeting the antigen-specific arm of the immune system against tumor cells. Moreno et al. have successfully used this approach in mice to retarget T cells against MTV-induced mammary adenocarcinomas *in vitro* (Moreno, 1995). In addition, bispecific antibodies targeting the MUC1 tumor antigen, expressed at high levels in a majority of human bile duct carcinomas (BDC), were able to inhibit the growth of these tumors in BDC-grafted SCID mice (Katayose,

1996). Other tumor types against which bispecific antibodies have demonstrated anti-tumor efficacy include ovarian carcinoma (Van Ravenswaay Claasen, 1993; Luiten, 1997), colon carcinoma (Beun, 1993), gastric carcinoma (Hombach, 1997), melanoma (Riedle, 1998), and refractory Hodgkin's Disease (Hartmann, 1997). Some studies have also demonstrated the efficacy of bispecific antibodies in the neutralization of lung metastases in animal models (Bakacs, 1995; Kroesen, 1995; Penna, 1996). Thus, bispecific antibodies and bispecific antibody derivatives, such as single chain Fv proteins, are a promising new option for anti-tumor immune therapy.

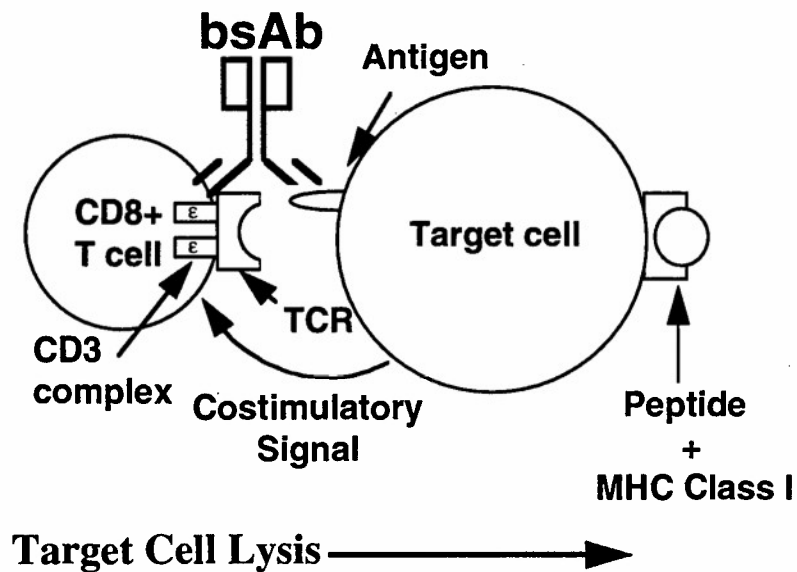
In conclusion, although investigators have encountered many challenges in the field of tumor immunotherapy, significant advancements have been made in this form of anti-tumor therapy. Both passive and active immunotherapeutic strategies have advantages and disadvantages, as described above, and the choice of an approach will depend on the particular therapeutic needs in a given situation. The study of immunotherapy has certainly improved our understanding of the immune system and how this complex and brilliantly designed system works. In addition, immunotherapy and concepts originating from the study of this field have changed the way we approach anti-tumor therapy. Hopefully, these advancements will translate into improved prevention of and survival from cancer in humans.

**Figure 1.5: Mechanism of bispecific antibody anti-tumor reactivity.** Bispecific antibodies effect tumor cell-killing by redirecting non-specific cytotoxic T lymphocytes to kill tumor cells bearing a target antigen for which the antibody is specific. Normally CD8+ T cells are activated to kill target cells following the interaction of a specific T cell receptor with a peptide fragment of the foreign antigen presented to the effector cell in the context of major histocompatibility complex proteins. A bispecific antibody bypasses the requirement for this specific TCR-peptide/MHC interaction by directly stimulating the T cell receptor when conjugated to an antigen on the surface of the target cell. Thus, any cytotoxic T cell can potentially be activated by a bispecific antibody to lyse any target cell expressing the appropriate antigen.

### Normal T cell activation:



### Activation by bispecific antibody:



## Adenoviral Gene Delivery

As we are beginning to discover the genetic basis of many disease processes, including cancer, the concept that the natural history of these diseases can be altered by genetic manipulation is becoming very popular. The challenges of gene delivery differ significantly for different disease processes; therefore, different delivery systems have been designed to meet particular needs. For example, correction of a defective gene, such as that encoding the cystic fibrosis transmembrane conductance regulator (CFTR) which is mutated in cystic fibrosis, requires a delivery system that will target enough cells to provide replacement of function. More importantly, however, this type of gene therapy requires delivery that will provide long term expression of the replaced gene, with minimal induction of an immunogenic response. Gene therapy for cancer has different requirements for several reasons. First, cancer cells are very genetically unstable and generally contain many different mutations by the time the patient presents for therapy, even if a single genetic defect was initially causally involved in tumorigenesis. Second, an important issue in cancer therapy is the need to treat tumor cells at the primary site as well as potential metastases, which complicates gene delivery as regards specificity as well as accessibility. Finally, in contrast to gene therapy for a disease with a defective gene, such as cystic fibrosis, every cancer cell must be "corrected" or killed, or the non-targeted tumor cells will grow out following treatment, resulting in relapse (Vile, 1995). For this reason, standard molecular therapeutic approaches to cancer treatment will likely have limited usefulness. However, gene therapy that targets cancer either via the immune system or via other normal systems that play a role in tumor development (such as blood vessels in angiogenesis) may provide a very effective means of eradicating malignancies.

Some "molecular" immunotherapeutic approaches have been discussed previously in this chapter, along with the advantages and disadvantages of these approaches.

Several different agents have been explored for *in vivo* gene delivery. Naked DNA injection has been tested, with some degree of success (reviewed in Wolff, 1997). However, this strategy requires that DNA uptake by the few cells surrounding the injection site be sufficient for the purposes of the therapy. Other non-viral delivery strategies, such as liposomes, are appealing because they have no limitations on the size of the gene they can carry, and there is minimal induction of the immune system against the replacement gene with liposomal delivery (Amalfitano, 1998). However, in practice such strategies have not been very successful at sufficient delivery of target genes (Zabner, 1995; Fasbender, 1997). Retroviral vectors have been more successful, in that these agents have been designed in nature to "deliver" genes, through infection of target cells. Retroviral gene delivery has proven very successful *in vitro*; however, its usefulness has been limited *in vivo* (reviewed in Gunzburg and Salmons, 1996). Unlike liposomes, retroviruses have a limited carrying capacity. In addition, retroviruses only infect actively proliferating cells, and many gene therapy strategies require targeting of non-proliferative, differentiated tissues. Finally, retroviral DNA integrates into the host genome, which is useful for long-term persistence of the inserted gene. However, the danger of oncogene activation or disruption of a tumor suppressor gene and resultant tumorigenesis makes *in vivo* administration of such an agent to human subjects a risky therapeutic option.

*In vivo* gene delivery by adenoviral vectors has many advantages as a means of genetic alteration of target cells. Adenovirus (Ad) has a relatively large genome of 36 kB and thus a relatively large potential carrying capacity, and its DNA is relatively easy to manipulate using recombinant techniques. In addition, it grows to high titer (up to  $10^{12}$  plaque forming units/ml) and infects a large fraction (80-100%) of a broad range of cell

types, including quiescent and differentiated cells, inducing a very high level of expression of the transgene. Finally, Ad is easy to store and rarely integrates into the host genome, making this a safer option for use in humans than retroviral gene delivery (Hitt, 1995; Spector and Samaniego, 1995). The safety of Ad as a gene delivery agent in human subjects is attested by the fact that live adenoviral vaccines administered to military recruits have not resulted in severe illnesses in these individuals (Amalfitano, 1998). Two of the most serious criticisms of the earliest adenoviral delivery vectors are their limited (but relatively substantive) carrying capacity of up to 8 kB and their immunogenicity. Of note, the inflammatory response induced by viral proteins may actually be advantageous in strategies involving the use of adenoviral vectors for cancer vaccines (Descamps, 1996). This issue will be discussed in more detail in chapter 5. However, for gene therapy strategies in which the goal is replacement of a defective gene, induction of an immune response limits the duration of expression of the gene of interest in host tissues and thus has been a major limiting factor for this type of therapy. This limitation is currently being addressed with the creation of second and third generation adenoviral gene delivery systems, which are less antigenic and additionally have increased cloning capacities (Hauser, 1997).

Adenoviruses are known clinically for causing upper respiratory infections characterized by fever, sore throat, runny nose, and conjunctivitis, as well as atypical pneumonia in the lower respiratory tract. Young children (< 2 years) may present with non bloody diarrhea due to gastrointestinal adenoviral infection, and hemorrhagic cystitis (bladder infection accompanied by blood in the urine) may also occur (Levinson and Jawetz, 1994).

The adenoviral genome consists of double stranded linear DNA packaged in an icosahedral nucleocapsid. This virus is non-enveloped and is unique in that it has a hemagglutinin fiber protruding from each of the 12 vertices of the capsid, which serve as

its point of attachment and entry into foreign cells. Different fiber types define the different strains of Ad, as these are the primary antigenic component of the virions (Levinson and Jawetz, 1994).

The replicative cycle of Ad begins with attachment to the target cell via its fiber, internalization by receptor-mediated endocytosis, and translocation of viral DNA to the nucleus. The host RNA polymerase transcribes the viral genome, beginning with the early genes, E1 - E4, which encode proteins required for DNA replication, viral propagation, and late gene expression. The late genes encode structural viral proteins. The virion then assembles in the nucleus of the cell, and the replicative cycle is terminated by lysis of the host cell, releasing the virion into the extracellular environment to infect another cell (Levinson and Jawetz, 1994).

Ad type 5 has been used most commonly in gene therapy strategies. The first generation gene delivery vectors are based on a mutant in which either the E1 or the E1 and E3 regions are replaced by the target gene of interest. These recombinant adenoviridae can be grown to high titers in the E1-complementing 293 human embryonic kidney cell line. When non-permissive cells are infected, the introduced gene is expressed at high levels if provided with its own promoter; however, the virus is replication-defective and thus non-pathogenic in the host (Kozarsky and Wilson, 1993; Spector and Samaniego, 1995). Although the E1 gene encodes regulatory proteins that are necessary for trans-activation of the other viral early and late promoters, low level expression of other early and late genes still occurs in E1-defective Ad infected cells, most likely due to endogenous "E1-like" activities in these cells (Amalfitano, 1996). Thus, in later generation delivery vectors, investigators have further pared components of the viral genome which are structurally unnecessary but are critical for viral replication in non-permissive cells. Three genes which have been targeted are encoded in the E2 region, and include the E2a gene product DPB, as well as products of the E2b gene, the



DNA polymerase (POL) and the preterminal protein (pTP) (Amalfitano and Chamberlain, 1997). However, the deletion mutants which can be constructed are limited by the need to grow these viruses in permissive cells which provide the missing gene products in trans. This may not be possible if expression of adequate amounts of the gene products is toxic for these cells, as has been shown for DPB (Klessig, 1984; Amalfitano, 1996). Recently Amalfitano *et al.* have described the generation of an [E1<sup>-</sup>, Δpol, E3<sup>-</sup>] Ad vector with a carrying capacity of 9.0 kB (theoretically approaching 11 kB, with further deletion of the polymerase gene) and decreased expression of viral late genes, including the immunogenic fiber protein (Amalfitano, 1998). Thus, as adenoviral vectors continue to be improved, the possibilities for gene therapy and/or high level expression of very large gene products are becoming more of a reality.

## Summary

Prevention of metastatic breast cancer is limited to early detection and surgical resection of a localized tumor. Treatment for advanced breast cancer is often unsuccessful due to micrometastasis of the tumor prior to therapy. Thus, new methods to treat and prevent metastatic breast cancer are sorely needed.

Prevention and treatment of breast cancer is advanced via an improved understanding of breast cancer biology. In subsequent chapters, I will describe data suggesting a potential role for the inherited breast cancer susceptibility gene product BRCA2 in normal cellular differentiation processes. These findings are important, as the elucidation of the role this protein plays in normal breast biology may help us to understand why tumorigenesis occurs in hereditary cases. In addition, these findings may be applicable to non-inherited breast cancer.

Metastatic breast cancer is often incurable using conventional forms of therapy. Thus, it is imperative that we explore novel therapeutic options, such as immunotherapy. EGFRvIII, a variant form of the epidermal growth factor receptor, is found in a large percentage of breast and brain tumors and thus may be a viable target for immunotherapy. In subsequent chapters, I will describe the development of an antibody bispecific for both EGFRvIII and a T-cell activation antigen, which can be used to redirect the cytotoxic response of T-cells with a broad range of specificities against breast cancer cells that express EGFRvIII. In addition, I will also describe the development of an EGFRvIII-transducing adenovirus which can be utilized in active immunotherapeutic protocols targeting the EGFRvIII tumor antigen.

## **Chapter 2**

# **Materials and Methods**

## **Antibodies.**

The anti-BRCA2 monoclonal antibody 2C9 and anti-BRCA2 polyclonal rabbit antiserum #5815 were generated in our laboratory as described later. Additional anti-BRCA2 mAbs generated against sequences in exon 14 (mAbs 5F6 and 9D3) or against a peptide fragment encoded by bp 792-1917, corresponding to mid exon 7 through most of exon 10 (mAb 3E6) were kind gifts of Dr. Wen Hwa Lee. Anti-cytokeratin (CK) mAbs AE1 and AE3 (reactive with CK 1-2, 5, 6, 14) (Woodcock-Mitchell, 1982) were obtained from Boehringer Mannheim (Indianapolis IN). Anti-CK mAb AE2 (reactive with CK 1, 2, and 10 found in terminally differentiated cells) (Woodcock-Mitchell, 1982) was a kind gift from Dr. Barton Haynes. Mib-1 mAb specific for the Ki-67 nuclear antigen expressed by all cells that are not in G<sub>0</sub> (Key, 1993) was obtained from Immunotech Inc. (Westbrook ME). Anti-wild type EGFR mAb Ab-1 (clone 528) was obtained from Oncogene Science (Cambridge MA) or was obtained from tissue culture supernatant, as described below. The anti-EGFRvIII mAb L8A4 (Wikstrand, 1995), as well as L8A4 RNA, were obtained from our collaborator, Dr. Darell Bigner. Anti-phosphotyrosine mAb 2G8.D6 and negative control mAb P3x63 were obtained from tissue culture supernatant, as described below.

## **Proteins and Peptides.**

BRCA2 proteins: The proteins used as an immunogens for anti-BRCA2 monoclonal and polyclonal antibody production were generated in the laboratory of our collaborator, Dr. Jeffrey Marks. In brief, BAC1 and BAC2, two non-overlapping 49 kDa protein fragments derived from BRCA2 exon 11 (bp 2716-3714 and 3748 to 4817, respectively,

Genbank U43746), were produced as glutathione S-transferase (GST)-fusion proteins in bacteria, cleaved from GST, and gel purified.

EGFRvIII peptide: Pep-3, the 14 amino acid peptide used in ELISA assays to test anti-EGFRvIII reactivity, was obtained from the laboratory of our collaborator, Dr. Darell Bigner. Pep-3 represents the portion of the EGFRvIII sequence which spans the novel fusion junction (underlined) and has the following sequence: LEEKKGNYVVT DHC (Wikstrand, 1995).

### **Generation of Anti-BRCA2 Antibodies.**

Monoclonal antibody 2C9 and mouse polyclonal BAC2 antisera: For generation of the monoclonal antibody 2C9, as well as anti-BAC2 mouse polysera, 50 µg of the purified BAC2 protein was emulsified with complete Freund's adjuvant (Sigma, St. Louis MO) and injected subcutaneously into each of three Balb/C mice (#4-6) (Charles River Laboratories, Wilmington MA). The immunizations were repeated at weeks two and four, using BAC2 in incomplete Freund's adjuvant (Sigma). At week six, one mouse (#5) was given a final intraperitoneal boost with BAC2 protein in PBS. Mouse #5 was sacrificed three days later, and hybridoma cells were generated according to standard methods (Kohler and Milstein, 1975; Searce and Eisenbarth, 1983) by Richard Searce in the laboratory of Dr. Barton Haynes. In brief, a polyethylene glycol (PEG)-induced fusion of the mouse splenocytes with P3x63 myeloma cells was performed, followed by selection for hybridomas in media containing HAT supplement (0.1 mM hypoxanthine;  $4 \times 10^{-5}$  mM aminopterin;  $1.6 \times 10^{-3}$  mM thymidine) (Gibco BRL, Grand Island NY). After two weeks in culture, an initial screen for BAC2 antibody-producing hybridomas was performed by ELISA. 66 positive clones (defined as having reactivity greater than 3.5-

fold over background) were cultured for an additional week, followed by a second screen for reactivity against the immunizing protein, BAC2. From this group, six clones maintained their reactivity against BAC2 (>2.5X over background). When screened against an irrelevant peptide (BAC1), one clone, **2C9**, was non-reactive and thus presumed to be specific for BAC2. 2C9 was then subcloned twice, generating the monoclonal antibody 2C9 Cl 1-1.

Polyclonal mouse antisera reactive with the purified BAC1 protein were also generated in three mice (#1-3), in a manner identical to that described above for anti-BAC2 sera. These anti-BAC1 polysera were used as a negative control in some experiments.

Polyclonal antiserum #5815: For the primary immunization, a strip of 10% polyacrylamide gel (totaling 5.5 cm<sup>2</sup> in surface area) containing the BAC1-GST fusion protein (of moderate concentration by Coomassie stain) was emulsified with complete Freund's adjuvant and injected subcutaneously into rabbit #5815 (Mayes Rabbit Ranch, Wilson NC). Booster immunizations were given, using 500 µg (week 3) and 400 µg (week 10) of BAC1-GST in incomplete Freund's adjuvant. At 6 months, rabbit #5815 was given a final boost with 500 µg of BAC1-GST protein in incomplete Freund's adjuvant. A terminal bleed was done nine days later. Serum from rabbit #5814, which did not receive any immunizations, was used as a negative control.

### **Tissues Derived from Human Subjects.**

Human Tissue: Samples of fresh human tissues were obtained as discarded tissues from the Department of Pathology. These tissues were used fresh, snap frozen in liquid nitrogen, or prepared for sectioning. For frozen sections, portions of each tissue were

embedded in Tissue-Tek O.C.T. compound (VWR Scientific Products, Atlanta GA) and snap frozen using PF5060 freezing fluid in a Histobath (Shandon Lipshaw, Pittsburgh PA) maintained at -60 to -70°C. Additional portions of tissue were formalin fixed for 8-24 hours then processed into paraffin blocks, using standard methods. 4 µm sections of either frozen or formalin-fixed paraffin-embedded (FFPE) tissues were placed on gelatin coated or Superfrost (Fisher Scientific, Pittsburgh PA) slides for further use in immunohistochemical assays.

Breast Cancer Patient Plasma: Coded samples of plasma from breast cancer patients and controls were obtained through the Tissue and Plasma Bank of the Duke University Specialized Program of Research Excellence (SPORE) in Breast Cancer (Drs. Jeffrey Marks, J. Dirk Iglehart).

Human Foreskin Keratinocytes: Human neonatal foreskins were obtained from the Duke University Medical Center newborn nursery in accordance with protocols for discarded tissue. The foreskins were first disinfected with four sequential washes in sterile Hanks Buffered Saline Solution (HBSS) without CaCl<sub>2</sub>, MgCl<sub>2</sub>, MgSO<sub>4</sub>, or phenol red (Gibco BRL). The epidermis was isolated by sectioning the tissue into strips of approximately 3x10 mm, removing most of the fatty portion of the dermis, and incubating overnight in trypsin solution (0.25%, 4°C), followed by mechanical removal of the epidermal layer with forceps. The epidermis was then placed into a 15 mL conical tube containing 5 mL trypsin neutralizing solution (HBSS + 15% fetal calf serum (FCS) (Hyclone, Logan UT)). Keratinocytes were brought into single cell suspension by pipetting up and down several times, and the cells were pelleted by centrifugation at 1500 rpm for 3 minutes. The pellets were then resuspended and cultured as described below.

## **Tissue Culture.**

Cells and Growth Media: MCF-7, BT483, SKBR3, ZR75-1, T47D, and DU4475 breast carcinoma cell lines, obtained from the American Type Culture Collection (ATCC) (Rockville, MD), were grown in RPMI 1640 medium (Gibco BRL) with 10% FCS or 20%FCS (DU4475). Other breast carcinoma cell lines were grown as follows: MDA-MB-231 (obtained from the ATCC), MDA-MB-361, and MDA-MB-435 (both obtained from MD Anderson, Houston TX) were grown in Minimal Essential Medium with Earle's Salts (Earle's MEM) (Gibco BRL) with 0.1 mM MEM Non-Essential Amino Acids Solution (Gibco BRL) and 10% FCS. The Cos-7 SV40-transformed African green monkey kidney cell line was obtained from the Duke University Tissue Culture Facility and was grown in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL) with 5% FCS. LP-293 embryonic kidney epithelial cells that constitutively express the adenoviral E1 gene, a kind gift from Dr. Andrea Amalfitano, were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL) with 10% FCS. B-6 cells generated by stable transfection of LP-293 cells with the adenoviral polymerase gene, a kind gift from Dr. Amalfitano, were grown in DMEM with 10%FCS and 100 µg/mL hygromycin B (Boehringer Mannheim). CRIP cells producing EGFRvIII-transducing retrovirus obtained from Dr. Glenn Dranoff (Danos and Mulligan, 1988) were grown in DMEM with 10% iron-supplemented calf serum (CS) (Hyclone). Most cells were cultured with the antibiotics penicillin G-sodium (1000 U/mL) and streptomycin-sulfate (1000 µg/mL) (Gibco BRL) as well as 2 mM L-glutamine (Gibco BRL).

Culture of Human Foreskin Keratinocytes: Human foreskin keratinocytes (HFKs), derived as described above, were grown in Keratinocyte Serum-free Medium (Gibco



BRL) in 150-cm<sup>2</sup> tissue culture flasks which had been coated overnight at 37°C with 4 mL FCS and rinsed with 10 mL HBSS prior to plating the keratinocytes. HFK primary cultures were subcultured into 75-cm<sup>2</sup> flasks at 70% confluence, and were thereafter passed at 70% confluence at 1:6, every three to four days.

Hybridoma Cells: The hybridoma cell line producing mAb 2C9 was derived as described above and was grown in DMEM with 10% Fetal Clone I serum (Hyclone). The hybridoma cell line producing anti-phosphotyrosine mAb 2G8.D6 was obtained from the ATCC and was grown in IMDM with 10% Fetal Clone I. The OKT3 (anti-CD3 $\epsilon$ ) and SP2/0 hybridoma cell lines obtained from the ATCC were grown in DMEM with 10% Fetal Clone I serum. The negative control antibody used in most experiments was the IgG1 mAb secreted by the myeloma line P3X63Ag8 (Kearny, 1979). This hybridoma, a kind gift from Dr. Barton Haynes, was grown in DMEM with 10% Fetal Clone I serum. The P3x63 myeloma line was also the fusion partner used for the generation of the hybridoma producing anti-BRCA2 mAb 2C9.

Culture Conditions: All cultured cells were grown at 37°C in 5% CO<sub>2</sub> (non-hybridoma cells) or 10% CO<sub>2</sub> (hybridoma cells).

Cell Passaging: Cells were passaged by rinsing once in Dulbecco's Phosphate Buffered Saline (DPBS) without Ca<sup>+2</sup> or Mg<sup>+2</sup> (Gibco BRL), followed by incubation in 0.05% trypsin/0.53 mM EDTA·4Na solution (Gibco BRL) for 5 minutes at 37°C. The trypsin was neutralized by addition of an equivalent amount of growth media containing serum (or HBSS + 10% serum for keratinocytes) and cells were passaged at 1:4-1:20, depending on the growth rate of the cells. B-6 and LP-293 cells were passaged by rinsing briefly

one time with 1X citric saline solution (134 mM KCl; 15 mM sodium citrate, dihydrate) which caused the cells to detach from the plate. These cells were then plated at 1:10.

### **Production of EGFRvIII-Expressing Adenovirus.**

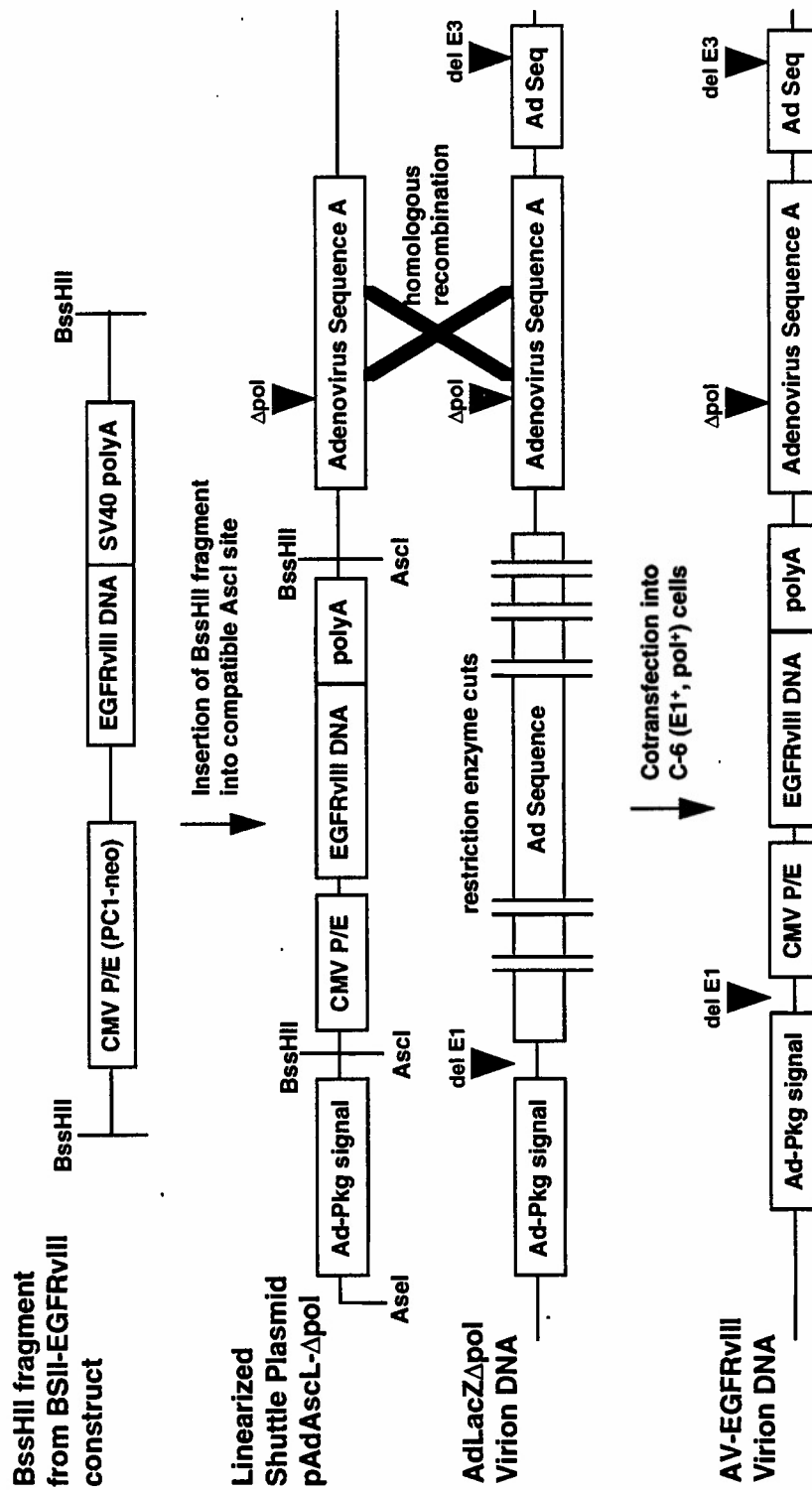
Construction of the EGFRvIII-containing adenoviral shuttle plasmid, pAdAscL- $\Delta$ pol-EGFRvIII, is described in Chapter 5. The recombinant EGFRvIII-producing adenovirus (Ad) was generated in the laboratory of Dr. Andrea Amalfitano, as described (Amalfitano, 1998) (Figure 2.1). Briefly, 40  $\mu$ L of infectious AdLacZ $\Delta$ pol virion DNA (0.41  $\mu$ g/ $\mu$ L) was fragmented by restriction enzyme digestion with XbaI, ClaI, and ScaI, followed by enzyme removal using a Sephadex spin column. The multiple enzyme digestion of the infectious virion DNA has been shown to greatly reduce the number of non-recombinant clones obtained (Amalfitano, 1998). One half of the purified Ad DNA and 60  $\mu$ L of AseI-linearized shuttle vector pAdAscL- $\Delta$ pol-EGFRvIII (0.72  $\mu$ g/ $\mu$ L) were co-transfected by calcium phosphate precipitation into a 60mm dish of C-7 cells, shown previously to effectively support the growth of Ads deleted for the E1 and polymerase genes (Amalfitano, 1996; Amalfitano and Chamberlain, 1997). The following day, the transfected cells were harvested, combined with an equal number of non-transfected C-7 cells, suspended in 60 mL of growth media, distributed evenly into 6 x 24 well dishes, and incubated at 37°C. One week later, five individual wells demonstrating viral cytopathic effects (CPE) were harvested, and these clones were amplified to determine whether they contained the recombinant gene. Amplification was performed in our laboratory as follows: prior to each infection, virions were released from the infected cells by three sequential freeze/thaw cycles. The cellular debris was pelleted by centrifugation, and 500  $\mu$ L of each virus-containing supernatant was used for the infection of 60mm dishes containing confluent B-6 cells. To infect the cells, growth media was

removed from the B-6 cells, the virus-containing supernatant was added, and the cells were incubated at 37°C for one hour, with gentle rocking every 15 minutes to ensure even distribution of the small volume of liquid. Growth media was then added back to the plates, and the infected cells were grown for 48-72 hours at 37°C, or until all the cells had lifted off the plate due to viral CPE. A second infection was performed using 1 mL of each secondary lysate to infect 60mm dishes containing confluent B-6 cells, and the infected cells were harvested after 24-30 hours of incubation at 37°C, followed by screening for recombinant EGFRvIII-producing clones as described in Chapter 5. Clone #5, which contained the recombinant gene, was then further amplified by repeated infection of B-6 cells in the following sequence: 1 x 150mm dish, 3 x 150mm dishes, 10 x 150mm dishes and finally 30 x 150mm dishes. After harvesting cells from the thirty 150mm dishes, the cell pellet was resuspended in 20 mL Homogenization Buffer (10 mM Tris, pH 7.4; 250 mM NaCl). Virus was released from the infected cells by three sequential freeze/thaw cycles and the cellular debris was pelleted at 2800 rpm x 15 minutes. Virus-containing supernatant was then used at volumes of 60 µL to 100 µL for infections of 100mm dishes.

## **Eukaryotic Cellular Transfection Methods.**

Transfection by Calcium Phosphate Precipitation: Cells plated in 100mm dishes were fed with 9.0 mL of growth medium 2 hours prior to the transfection. 10 µg of DNA was diluted with dH<sub>2</sub>O in a total volume of 450 µL, followed by the addition of 50 µL of 2.5 mM CaCl<sub>2</sub>. The DNA/CaCl<sub>2</sub> complexes were then added dropwise to 500 µL of 2X HEPES-buffered saline solution (HeBS) (0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.05), while expelling air bubbles in the HeBS with a pasteur pipette. The precipitate solution was immediately vortexed for 5 seconds and then incubated for 20

**Figure 2.1: Generation of an EGFRvIII-transducing adenovirus.** CMV promoter and enhancer, the EGFRvIII coding sequence (Genbank #X00588), and SV40 polyA sequences were first assembled in the BSII cloning vector, as diagrammed in Figure 5.2, followed by insertion into the adenoviral shuttle vector, pAdAscL- $\Delta$ pol, as diagrammed in Figure 5.4. The EGFRvIII-containing shuttle vector was then cotransfected into C-7 complementing cells along with fragmented AdLacZ $\Delta$ pol virion DNA. The C-7 cell line, a derivative of the LP-293 E1-complementing cell line which has been additionally stably transfected with the polymerase and pTP genes, is capable of transcomplementing E1-negative Ads, as well as Ad mutants with polymerase and pTP deletions (Amalfitano and Chamberlain, 1997). Adenoviral sequences in the shuttle vector undergo homologous recombination with virion DNA to produce a recombinant adenoviral genome containing the CMV promoter and enhancer, EGFRvIII sequence, and polyA sequence.



minutes at room temperature. Next, the precipitate solution was pipetted over the cells and gently rocked to distribute evenly. Following a 5 hour incubation at 37°C, the medium was removed, 2 mL of sterile 12% glycerol solution was added, and the plates were incubated for 2 minutes at room temperature. To remove the glycerol solution, the plates were washed three times with 5 mL of DPBS without  $\text{Ca}^{+2}$  or  $\text{Mg}^{+2}$ . The cells were then fed with standard growth media and grown as per usual method.

Transfection Using Commercially Available Transfection Reagents: Lipofectamine Plus (Gibco BRL), Transfectam (Promega, Madison WI), DOTAP (Boehringer Mannheim), and Eugene 6 (Boehringer Mannheim) were used according to the manufacturers' protocols.

Retroviral Transduction: CRIP cells producing retrovirus transducing the EGFRvIII or wild-type EGFR genes were plated in 100mm dishes at 1:2 to 1:3 and grown as previously described. Target cells were also plated in 100mm dishes and grown as previously described. Once the CRIP cells reached confluence (day 2), 10 mL of fresh medium was added, and the CRIP cells were incubated for another 24 hours. On day 3, the 24 hour CRIP medium containing retrovirus was removed and sterile filtered (0.45  $\mu\text{m}$ ). The 24 hour CRIP media was then combined with 10 mL plain DMEM and 30  $\mu\text{L}$  Lipofectamine (Gibco BRL); this mixture was incubated at room temperature for 30 minutes. The standard growth medium was removed from the target cells (now at 50-70% confluency), and the cells were incubated with the retroviral transfection mixture for 24 hours at 37°C in 5%  $\text{CO}_2$ . On day 4, following the 24 hour transfection, the retroviral-containing medium was removed, and the target cells were passaged into fresh 100mm dishes in standard growth medium. This procedure was repeated 8-10 times to ensure high level transfection with the gene of interest.

Adenoviral Transduction: Adenoviruses obtained as described above were added to target cells and incubated for one hour at 37°C, with gentle agitation every 15 minutes. Standard growth media was then added to the target cells, and the cells were incubated at 37°C in 5% CO<sub>2</sub> for 24-48 hours.

### **Enzyme Linked Immunosorbant Assay (ELISA).**

EIA/RIA plates (Corning Costar, Cambridge MA) were coated with 0.25-0.5 µg peptide (BAC1, BAC2, or Pep-3) diluted in CBC Buffer (15mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, 3 mM NaN<sub>3</sub>) and incubated overnight at 4°C. The wells were then blocked with 200 µL CBC + 3% Bovine Serum Albumin (BSA) (Sigma) for 2 hours at room temperature and washed three times with Wash Buffer (Phosphate Buffered Saline + 0.05% Tween-20). The wells were incubated with primary antibody (100 µL of tissue culture supernatant or 100 µL of serum diluted 1:100) at room temperature for 90 minutes. The wells were then washed three times with Wash Buffer and one time with dH<sub>2</sub>O, and 100 µL of alkaline-phosphatase conjugated goat anti-mouse or anti-rabbit IgG (Promega, Madison WI), diluted 1:6000, was added to the wells. The plate was incubated for 60 minutes at room temperature, followed by washes as described above. Then, 100 µL of 1 mg/mL p-Nitrophenyl phosphate substrate (Sigma) dissolved in substrate buffer (15mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>) was added, and the plates were incubated covered for 60 min at room temperature. Color development was measured by determining absorbance at 405nm.

## **Immunohistochemistry.**

4  $\mu$ m frozen tissue sections were acetone fixed and blocked with 5% goat serum for 20 minutes at 37°C. The sections were reacted with primary antibody for 30 minutes at 37°C, followed by a 10 minute wash with DPBS. The bound antibodies were then reacted with biotinylated horse anti-mouse or goat anti-rabbit IgG (Vector Laboratories, Burlingame CA) for 30 minutes at 37°C, followed by a wash as described above. Finally, the sections were incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain Elite ABC Kit, Vector Laboratories) for 30 min at 37°C, washed, and then incubated with 0.5 mg/mL 3,3'-diaminobenzidine (DAB)/H<sub>2</sub>O<sub>2</sub> substrate solution (Sigma) for 5 minutes. Sections were washed, counterstained with hematoxylin, and permanently mounted. To enhance sensitivity in some experiments, signals were amplified by catalyzed signal reporter deposition (Kerstens, 1995) using the Tyramide Signal Amplification (TSA) system (NENT<sup>TM</sup> Life Sciences Products, Boston MA) according to the manufacturers instructions. Where indicated, formalin-fixed paraffin-embedded tissue sections were deparaffinized, then heated in Glyca antigen retrieval solution (BioGenex, San Ramon CA) for 2C9 or 0.01M citrate solution, pH 6.0, for cytokeratins, 2 x 5 minutes at 600 Watts in a microwave oven, prior to the blocking step and further immunostaining as described above. For immunohistochemical analysis of cultured cells, cells were plated on glass slides or Lab-Tek Chamber Slide Culture Chambers (Nalge Nunc International, Milwaukee WI) and grown to 70-80% confluence. The slides were then rinsed with PBS, acetone fixed (80% acetone for chamber slides, 100% acetone for glass slides), and stained as described above for frozen sections.



## **Immunofluorescence.**

Frozen tissue sections were acetone fixed and rinsed 3 x 5 minutes in PBS. The sections were incubated with supernatants of monoclonal antibody 2C9 or negative control antibody P3x63 for 30 minutes at room temperature, followed by washes as described above. The sections were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg MD) for 30 minutes at room temperature, followed by washes. The slides were mounted and viewed under a fluorescence microscope.

## **Western Blot Analysis.**

Protein extracts were prepared using 10 mM Tris, 150 mM NaCl, and 1% Triton X-100, pH 8.0, in the presence of proteinase inhibitors (20 mM iodoacetamide, 0.02 mM E64, 0.03 mg/mL aprotinin, 0.07 mM pepstatin A, 1 mg/mL antipain, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM N $\alpha$ -p-tosyl-L-lysine chloromethyl ketone (TLCK)) or Complete Protease Inhibitor Cocktail Tablets, EDTA-free (Boehringer Mannheim) at a concentration of 50  $\mu$ L extraction buffer per 10 $\times$ 10<sup>6</sup> cells. Urea extraction buffer (8M urea, 0.1M NaH<sub>2</sub>PO<sub>4</sub>; 0.1M Tris, pH 8.0) was used instead of standard extraction buffer in some experiments as a means of achieving complete solubilization of cytokeratins. Following a 20 minute incubation on ice, samples were centrifuged for 30 minutes at 14,000 rpm, 4°C to remove insoluble material. Protein concentrations in cellular lysates were determined by reaction with Cu<sup>+2</sup> and bicinchoninic acid (BCA), using the BCA Protein Assay Reagent (Pierce, Rockford IL), according to the manufacturer's instructions. Lysates were boiled in 2X

SDS sample buffer (0.5M Tris-HCl, pH 6.8; 10% SDS; 20% glycerol; 0.1% bromophenol blue) for 5-10 minutes, separated on a 4% or 5% sodium dodecyl sulfate (SDS) polyacrylamide gel, or a 4-20% Tris-HCl Ready Gel (Bio-Rad, Hercules CA), and transferred to nitrocellulose. Blots were then incubated with 10% nonfat dry milk in Tris buffered saline + 0.05% Tween 20 (TBST) to block non-specific reactivity, then with primary antibody, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc, West Grove PA), with intervening washes. Alternatively, HRP-conjugated goat anti-human F(ab')<sub>2</sub> (Jackson ImmunoResearch Laboratories) was used to directly detect protein products of bispecific antibody constructs, followed by washes. Antibody-bound protein was visualized by exposure to a chemiluminescent substrate (ECL Western blotting protocol, Amersham Life Science, Arlington Heights, IL; Western Blot Chemiluminescence Reagent Plus, DuPont NEN, Boston MA; or Supersignal Substrate, Western blotting protocol, Pierce, Rockford IL) and detection on Biomax MR film (Eastman Kodak Company, Rochester NY).

### **Immunoprecipitation.**

Lysates prepared as described above were precleared twice with 20  $\mu$ L of control P3x63 supernatant, followed by 20  $\mu$ L of goat anti-mouse IgG-agarose (Sigma). BRCA2 protein was then immunoprecipitated with 20  $\mu$ L of 2C9 supernatant, followed by 20  $\mu$ L of goat anti-mouse IgG-agarose. For all reactions with primary and secondary antibodies (except 2C9), lysates were incubated for one hour at 4°C with end-over-end mixing; lysates were incubated with 2C9 antibody overnight at 4°C with end-over-end mixing. Following immunoprecipitation, the agarose pellets were washed sequentially with 500  $\mu$ L of the following solutions: 1) TSA (0.01 M Tris, pH 8; 0.14 M NaCl; 0.025% NaN<sub>3</sub>) + 0.1%

Triton X-100; 2) TSA + 0.1% Triton X-100; 3) TSA; and 4) 0.05 M Tris-Cl, pH 6.8. Cells were gently pelleted (450 rpm for 5 minutes) after each wash. The pellets were then boiled in SDS sample buffer for 5-10 minutes, the agarose was pelleted, and the supernatant was analyzed by Western blot as described above.

### **Flow Cytometric Analysis.**

Cells to be analyzed were brought into suspension by mechanical dissociation and/or trypsinization, dispensed into sorter tubes (Falcon #2052, Becton Dickinson Labware, Lincoln Park NJ) at a density of  $2 \times 10^5$  epithelial cells per tube, and pelleted by gentle centrifugation at 1200 rpm, 5 minutes, 4°C. The cells were rinsed 1 x in Azide Wash Solution (1% BSA and 0.1% Sodium Azide in DPBS), and the Azide Wash was decanted, leaving the cell pellet in a volume of approximately 50 µL. 50 µL of primary antibody, diluted in Azide Wash at 2-fold the concentration normally used to stain tissue sections, was added to the resuspended cell pellet and incubated for 30 minutes at 4°C. The cells were then washed 2 x with Azide Wash, leaving the cell pellet in a volume of approximately 50 µL after the final wash. 50 µL of FITC-conjugated goat anti-mouse IgG (H+L) (Kirkegaard & Perry Laboratories), diluted 1:10 in Azide Wash, was added to each tube, followed by a 30 minute incubation at 4°C. The cells were again washed 2X with Azide Wash. After the final wash, the cells were brought up in 400-500 µL Azide Wash and fixed with 100 µL of 2% paraformaldehyde. Fluorescence of individual cells at 525 nm was measured at the Duke University Cancer Center Flow Cytometry Facility.

## **RNA extraction.**

Frozen sections: Two 20-micron frozen sections were collected in a microfuge tube. The following solutions were added sequentially, followed by vortexing after each addition: 1) 400  $\mu$ L of Denaturing Solution (4M guanidine isothiocyanate; 25 mM sodium citrate, pH 7; 0.5% n-lauryl sarcosine; 0.1 M  $\beta$ -mercaptoethanol); 2) 40  $\mu$ L of 2M sodium acetate, pH 4.0; 3) 440  $\mu$ L of acid phenol:chloroform mixture (5:1), pH 4.7; and 4) 40  $\mu$ L of chloroform:isoamyl alcohol mixture (24:1). The samples were then vortexed for 10 seconds, followed by chilling on ice for 15 minutes. Aqueous and organic phases were separated by centrifugation at 14,000 rpm, 4°C, for 15 minutes. The aqueous phase was then transferred to a fresh microfuge tube, making certain to avoid the interface, and 1  $\mu$ L glycogen (Boehringer Mannheim) was added as carrier, followed by 500  $\mu$ L isopropanol. Samples were placed at -20°C for at least 1 hour, and precipitated RNA was pelleted by centrifugation at 14,000 rpm, 4°C, for 10 minutes. The supernatant was removed, and the pellet was redissolved in 300  $\mu$ L Denaturing Solution. RNA was again precipitated by adding 300  $\mu$ L isopropanol, vortexing, and incubating at -20°C for at least 1 hour. Precipitated RNA was collected by centrifugation as described above. Finally, the pellets were washed with 300  $\mu$ L of 75% EtOH, briefly dried under vacuum, resuspended in 15-25  $\mu$ L RNase-free dH<sub>2</sub>O, and quantitated by determining absorbance at 260 and 280 nm.

Cultured Cells: Adherent cells grown in 75-cm<sup>2</sup> tissue culture flasks were rinsed once with cold DPBS and lysed directly in the flasks with 3.8 mL GIT Denaturing Solution (4M guanidium isothiocyanate; 25 mM sodium citrate, pH 7; 0.5% n-lauryl sarcosine; 0.1 M  $\beta$ -mercaptoethanol). The lysate was then transferred to a 15 mL conical tube. Suspension cells were pelleted by centrifugation in a 15 mL conical tube, washed once

with cold DPBS, and lysed with 3.8 mL GIT solution. Following addition of GIT, the lysates were vortexed vigorously for 2 minutes (until the solution was no longer viscous) and stored on ice. RNA was then isolated by ultracentrifugation of the lysate over a cesium chloride cushion. In brief, the GIT lysate was carefully layered on top of 1.2 mL of Solution B (5.7 M Cesium Chloride; 25 mM sodium acetate, pH 5.0; 0.1 M  $\beta$ -mercaptoethanol) in polyallomer centrifuge tubes (Beckman Instruments, Inc., Palo Alto CA). The samples were ultracentrifuged for 7.5 to 12 hours at 18°C, 36,000 rpm, in a Sorvall AH-650 rotor. RNA pellets were harvested in 100-200  $\mu$ L RNase free dH<sub>2</sub>O, heated to 56°C for 15 minutes, and stored at -20°C. Alternatively, the RNeasy Mini Kit (Qiagen, Valencia CA) was used according to the manufacturer's instructions.

Tissues: Fresh or frozen tissues were manually dissected into small pieces and ground in GIT solution using a mechanical tissue grinder (Tissue Tearor, Biospec Products, Inc. from Fisher Scientific, Norcross GA). RNA was then isolated as described for cultured cells.

### **Northern Blot Analysis.**

RNA samples were prepared for electrophoresis as follows: 10-20  $\mu$ g RNA was vacuum dessicated and combined with 5  $\mu$ L formamide, 2  $\mu$ L formaldehyde, 2  $\mu$ L dH<sub>2</sub>O, and 1  $\mu$ L of 10X MOPS (0.4 M 3-(N-morpholino)-propanesulfonic acid, pH 7.0; 0.1 M sodium acetate; 0.01 M EDTA). The RNA was denatured by heating to 65°C for 10-15 minutes, followed by addition of 1.1  $\mu$ L 10X standard loading buffer (1 mM EDTA, pH 8.0; 0.25% bromophenol blue; 0.25% xylene cyanol; 50% glycerol). RNA was separated by agarose/formaldehyde gel electrophoresis (1.2% agarose; 2.2 M formaldehyde), in MOPS

running buffer, for approximately 16 hours at 24 volts. Nucleic acids were denatured by shaking for 15 minutes in Northern Denaturing Solution (50 mM NaOH; 10 mM NaCl), followed by neutralization 2 x 15 minutes in Northern Neutralizing Solution (10X SSC [1.5 M NaCl; 0.15 M Na Citrate], 0.1 M Tris, pH 7.4). RNA was transferred overnight to a nitrocellulose membrane (Costar BioBlot, Corning Inc., Acton MA) by upward capillary transfer in a high salt buffer, using 10X SSC. The blot was baked in a vacuum oven at 80°C for 2 hours, briefly wet with dH<sub>2</sub>O, and incubated in formamide prehybridization/ hybridization (FPH) solution (5X SSC; 5X Denhardt solution [0.1% Ficoll-400; 0.1% polyvinylpyrrolidone; 0.1% BSA]; 50% formamide; 1% SDS; 100 µg/mL denatured salmon sperm DNA) for at least 4 hours at 42°C. A <sup>32</sup>P-labeled DNA probe was generated using the Prime-It II Random Primer Labeling kit (Stratagene, La Jolla CA), or the Random Primed DNA Labeling kit (Boehringer Mannheim) according to the manufacturers' instructions. The blot was then incubated overnight at 42°C with <sup>32</sup>P-labeled DNA probe, diluted at 10<sup>6</sup> cpm/mL in FPH solution. Following hybridization, the blot was washed as follows: Northern Wash #1 (2X SSC; 0.1% SDS) 4 x 5 minutes at room temperature; Northern Wash #2 (0.1X SSC; 0.1% SDS) 2 x 15 minutes at 55°C. Specific bands were visualized by autoradiography.

### **Reverse Transcription.**

2 µg of total RNA was combined with 100 ng random hexamer primers (Gibco BRL) and 1 µL RNasin Ribonuclease Inhibitor (Promega, Madison WI) in a total volume of 10 µL. This mixture was incubated at 68°C for 10 minutes, followed by cooling to 4°C. The following reagents were then added: 4 µL 5X 1st Strand Buffer (Gibco BRL), 1 µL of 10 mM dNTP (Gibco BRL), 2 µL of 0.1 M DTT (Gibco BRL), and 1 µL dH<sub>2</sub>O. The samples were brought up to 37°C, followed by the addition of 2 µL of the Superscript RNA-

dependent DNA-polymerase (Gibco BRL). cDNA was generated by reaction at 37°C for 15 minutes, 43°C for 60 minutes, and 98°C for 5 minutes, followed by 4°C. The cDNA was used immediately for PCR, or stored at -80°C.

### **DNA extraction.**

Cells were harvested, washed with cold PBS, and resuspended in DNA extraction buffer (10 mM Tris, pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5% SDS) with 0.3 mg/mL Proteinase K (Sigma) at a volume of 1 mL extraction buffer/ $10^8$  cells. The lysate was incubated at 55°C for 15 hours. Two phenol/chloroform/isoamyl alcohol (25:24:1) extractions were performed, followed by ethanol precipitation and centrifugation. The DNA pellet was rinsed with 70% ethanol, air dried, and resuspended in 100-200  $\mu$ L TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). Alternatively, DNA was extracted using the Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis MN) according to the manufacturer's instructions.

### **Southern Blot Analysis.**

DNA was separated by 0.8%-2% agarose gel electrophoresis, in TAE (40 mM Tris-acetate, 1 mM EDTA) running buffer. Separated DNAs were denatured by shaking for 30 minutes in 250 mL Southern Denaturing Solution (1.5 M NaCl; 0.5 N NaOH), followed by neutralization with two 30 minute washes in 250 mL Southern Neutralization Solution (0.5 M Tris, pH 8.0, 1.5 M NaCl). DNA was transferred overnight to a Nylon membrane (Boehringer Mannheim) as described for transfer of RNA in Northern blot analysis. The next morning, the blot was rinsed briefly with 5X SSC and the DNA was immobilized by UV irradiation. Southern blot analysis was then performed using

oligonucleotide probes labelled with the non-isotopic Oligonucleotide 3' End Labeling Kit (Florescein ddUTP) (DuPont NEN, Boston MA), according to the manufacturer's instructions. In brief, the membrane was prehybridized by incubation for 1 hour at a selected probe-dependent temperature (58.5°C for EGFRvIII) in prehybridization/hybridization buffer containing 0.5% blocking reagent (DuPont NEN), 5% dextran sulfate, and 0.1% SDS in 5X SSC. 100 pmol of oligonucleotide probe (in volume of 4 µL) was end-labeled with 4 µL of 10 nmol fluorescein-12-ddUTP by reacting with the following for 60 minutes at 37°C: 2.0 µL calf thymus terminal deoxynucleotidyl transferase (TdT) (0.1 units), 12.5 µL terminal transferase reaction buffer, 2.5 µL cobalt chloride, and 10 µL dH<sub>2</sub>O. The membrane was then hybridized overnight at 58.5°C with the labeled probe, diluted to 10 pmol/mL in P/H buffer. Washes were performed as follows: one wash with 250 mL Wash Buffer A (2X SSC, 0.1%SDS) at 58.5°C for 10 minutes; one wash with 250 mL Wash Buffer B (0.9 M NaCl, 0.1% SDS) at 58.5°C for 10 minutes; and 4-5 quick rinses with Buffer 1 (100 mM Tris-HCl, 150 mM NaCl) at room temperature to remove residual detergent. The membrane was then blocked in 20 mL Buffer 2 (Buffer 1 with 0.5% blocking reagent) for 1 hour at room temperature. Antibody conjugate solution (antifluorescein-HRP conjugate) was diluted at 1:1000 in Buffer 2 and incubated with the membrane for 1 hour at room temperature, followed by 4 x 5 minute washes in Buffer 1. Antibody-bound fluorescein-labeled DNA probe was visualized by exposure to a chemiluminescent substrate (Enhanced Luminol Reagent and Oxidizing Reagent (DuPont NEN), mixed in equal volumes) and detection on film.



## **DNA Manipulation.**

Vectors and Constructs: The following vectors were used for construction of the bispecific antibody and adenoviral constructs, as described in chapters 4 and 5: PCR-II (TA Cloning Kit, Invitrogen, Madison WI), PC1-neo (Promega, Carlsbad CA), pcDNA3.1/Hygro (Invitrogen, Madison WI), pSV2ΔHgpt-HuG1, pSV184ΔHneo-HuK (kind gifts of Dr. Sherie Morrison), pHβApr-1-neo-EGFRvIII expression construct (kind gift of Dr. Darell Bigner), and pAdAscL-Δpol adenoviral shuttle plasmid (kind gift of Dr. Andrea Amalfitano) (Amalfitano, 1998).

BRCA2 Construct: A cDNA containing BRCA2 bp 23-10502, encoding full length BRCA2 protein (translated from bp 229 to bp 10485) was created by splicing partial cDNAs derived from an MCF-7 breast carcinoma cDNA library (bp 229-9783) and polymerase chain reaction (PCR) cloning (bp 9784 to 10502). Generation of the final construct pBSX/BRCA2 was accomplished by cloning the BRCA2 cDNA into the pBSX/CMV/pA expression vector. This vector, a kind gift of Dr. Andrea Amalfitano, is based on the Bluescript vector (Stratagene), but has been modified to include the CMV immediate early promoter and enhancer and SV40 poly A sequences, to achieve high level expression of added cDNA sequences.

DNA Probes: The EGFR-specific DNA probe used for Southern blot analysis was a 293 bp EcoRI fragment derived from the 3' portion of the gene (bp 187-479) (Genbank sequence #X00588). The BRCA2-specific DNA probe used for Northern blot analysis consisted of a mixture of two EcoRI fragments of approximately 500 bp each, derived from BRCA2 exon 10 (bp 814-1329) and BRCA2 exon 11 (bp 1708-2207).

Polymerase Chain Reaction (PCR): For most reactions, the hot start method of polymerase chain reaction was used. For reactions amplifying cDNA products, PCR was performed using 2  $\mu\text{L}$  of cDNA in a total reaction volume of 75  $\mu\text{L}$ . First, 2  $\mu\text{L}$  each of 3' and 5' primers (from 20  $\mu\text{M}$  stock), 1.75  $\mu\text{L}$  of dNTP mix (from 10 mM stock), 2.5  $\mu\text{L}$  10X PCR Buffer (Boehringer Mannheim or Gibco BRL) with  $\text{Mg}^{+2}$ , and 16.75  $\mu\text{L}$   $\text{dH}_2\text{O}$  were combined in HotStart storage and reaction tubes (Molecular BioProducts, San Diego CA) and the mixture was heated to 80°C for 5 minutes. After cooling to 4°C, 48  $\mu\text{L}$  of the following mixture was added over the wax layer: 5  $\mu\text{L}$  10X PCR Buffer with  $\text{Mg}^{+2}$ , 44.5  $\mu\text{L}$   $\text{dH}_2\text{O}$ , and 0.5  $\mu\text{L}$  Taq DNA Polymerase (5 U/ $\mu\text{L}$ ) (Boehringer Mannheim or Gibco BRL). cDNA template was then added, and amplification cycles were performed, according to parameters determined for each primer set. For construction of the bispecific antibody, the Expand High Fidelity PCR System (Boehringer Mannheim) was used to minimize PCR-related errors in sequences. 2  $\mu\text{L}$  of dNTP mix (10 mM), 1.5  $\mu\text{L}$  each of 3' and 5' primers (20  $\mu\text{M}$ ), 42.5  $\mu\text{L}$   $\text{dH}_2\text{O}$ , and 2.5  $\mu\text{L}$  template cDNA or plasmid DNA were added to hot start tubes and the mixture was heated to 80°C for 5 minutes. After cooling to 4°C, 50  $\mu\text{L}$  of the following mixture was added over the wax layer: 39.25  $\mu\text{L}$   $\text{dH}_2\text{O}$ , 10  $\mu\text{L}$  10X PCR Buffer with  $\text{Mg}^{+2}$ , and 0.75  $\mu\text{L}$  Expand (3.5 U/ $\mu\text{L}$ ). Amplification cycles were performed, according to parameters determined for each primer set. Fusion PCR reactions used in construction of the bispecific antibody utilized a third overlapping (OL) primer, which was included in the reaction mixture at a much lower concentration than that of the 3' and 5' primers (1:1000, or 0.02  $\mu\text{M}$ ). Otherwise, this PCR reaction was performed in an identical manner to that described above. Sequences of custom oligonucleotide primer sets and PCR conditions used are shown in Figure 2.2. PCR-generated fusion sequences of the bispecific antibody constructs are shown in Figure 2.3.

**Figure 2.2: Primer sequences and polymerase chain reaction conditions.** Custom oligonucleotide primers were ordered chemically synthesized from Gibco BRL and used at a starting concentration of 20  $\mu$ M, with the exception of overlap (OL) primers, which were used at a starting concentration of 0.02  $\mu$ M. All primer sequences are shown in a 5' to 3' orientation.

## EGFR and EGFRvIII

### Amplification of EGFR and EGFRvIII-specific products:

5' EGFR                      GGG GAA TTC GCG ATG CGA CCC TCC GGG  
3' EGFR                      GGG AAG CTT TCC GTT ACA CAC TTT GCG

PCR: 1) 1 min, 20 sec 95°C; 2) 1 min 52°C; 3) 2 min 72°C; 4) 39 x to 1; 5) 8 min 72°C

## Bispecific Antibody

### Variable Region Cloning:

1) OKT3 heavy chain - Underlined sequence = EcoRI site; Bold sequence = Kozac Seq.

DKT3-2 5' HEAVY    GGA ATT CAC CAT GGA AAG GCA CTG GAT C  
OKT3 3' HEAVY    CGA ATT CTG AGG AGA CTG TGA GAG TGG T

2) OKT3 light chain - Underlined sequence = HindIII site; Bold sequence = Kozac Seq.

DKT3 5' LIGHT      CCT CAA GCT TCA AAA TGG ATT TTC AAG TGC  
                                 AG  
DKT3 3' LIGHT      CGT AAA GCT TGG TTT ATT TCC AAC TTT GTC CC

3) L8A4 heavy chain - Underlined sequence = EcoRI site; Bold sequence = Kozac Seq.

L8A4 5' HEAVY      GGA ATT CAC CAT GGG ATG GAG CTG GAT C  
L8A4 3' HEAVY      CGA ATT CAG CAG ATC CAG GGG CCA GTG G

4) L8A4 light chain - Underlined sequence = HindIII site; Bold sequence = Kozac Seq.

L8A4 5' LIGHT      CGT TAA GCT TCA CCA TGA GTC CTG CCC AGT T  
L8A4 3' LIGHT      CCT CAA GCT TTG GTG GGA AGA TGG ATA CAG T

PCR: 1) 1 min 94°C; 2) 1 min 60°C; 3) 45 sec 72°C; 4) 29 x to 1; 5) 8 min 72°C

### Construction of Fos and Jun sequences:

1) Fos sequence

Fos A 5'                      TTA ACT GAT ACA CTC CAA GCG GAG ACC GAC  
                                 CAG CTG GAA GAT AAG  
Fos A 3'                      GCA ATC TCG GTC TGC AGA GCA GAC TTC TTA  
                                 TCT TCC AGC TGG TCG  
Fos B 5'                      TGC TCT GCA GAC CGA GAT TGC CAA CCT GCT  
                                 GAA GGA GAA GGA AAA  
Fos B 3'                      TCA GGC GGC CAG GAT GAA CTC CAG TTT TTC  
                                 CTT CTC CTT CAG CAG

## 2) Jun sequence

Jun A 5'	CGC ATC GCC CGG CTC GAG GAA AAA GTG AAA ACC TTG AAA GCT CAG
Jun A 3'	GGC CGT GGA CGC GAG CTC CGA GTT CTG AGC TTT CAA GGT TTT CAC
Jun B 5'	GGA GCT CGC GTC CAC GGC CAA CAT GCT CAG GGA ACA GGT GGC ACA
Jun B 3'	TCA GTT CAT GAC TTT CTG TTT AAG CTG TGC CAC CTG TTC CCT GAG

PCR: 1) 1 min 94°C; 2) 1 min 56°C; 3) 1 min 72°C; 4) 29 x to 1; 5) 8 min 72°C

### Construction of IgG1-Fos and IgG-Jun fusion sequences

#### 1) Common 5' Primer - beginning of CH1 region

IgG1-5'	CCT CCA CCA AGG GCC CAT CG
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#### 2) IgG1-Fos Fusion Primers - Underlined sequence = XbaI site

IgG1-Fos OL2	CGC TTG GAG TGT ATC AGT TAA CCC GCC TGC TGA GGA AGA GAT GGA GG
IgG1-Fos-3'	CG <u>TCT AGA</u> TCA GGC GGC CAG GAT GAA CTC

#### 3) IgG1-Jun Fusion Primers - Underlined sequence = XbaI site

IgG1-Jun OL2	CTC GAG CCG GGC GAT GCG CCC GCC TGC TGA GGA AGA GAT GGA GG
IgG1-Jun 3'	CG <u>TCT AGA</u> TCA GTT CAT GAC TTT CTG TTT AAG

PCR: 1) 2 min 94°C; 2) 2 min 59°C; 3) 2 min 70°C; 4) 29 x to 1; 5) 8 min 70°C;

### Construction of fusion L8A4/IgG1-Jun and OKT3/IgG1-Fos sequences:

#### 1) L8A4/IgG1-Jun Fusion Primers

L8A4 5' HEAVY	GGA ATT CAC CAT GGG ATG GAG CTG GAT C
L8A4/IgG1Jun-OL2	ACT GGC CCC TGG ATC TGC TGC CTC CAC CAA GGG CCC A
IgG1-Jun 3'	CG TCT AGA TCA GTT CAT GAC TTT CTG TTT AAG

PCR: 1) 2 min 94°C; 2) 30 sec 94°C; 3) 1 min 62°C; 4) 1 min 72°C; 5) 2 x to 2; 6) 30 sec 94°C; 7) 1 min 58°C; 8) 1 min 72°C; 9) 4 x to 6; 10) 30 sec 94°C; 11) 1 min 65°C; 12) 1 min 72°C; 13) 21 x to 10; 14) 7 min 72°C

## 2) OKT3/IgG1-Fos Fusion Primers

DKT3-2 5' HEAVY GGA ATT CAC CAT GGA AAG GCA CTG GAT C  
OKT3/IgG1Fos-OL2 CTC TCA CAG TCT CCT CAG CCT CCA CCA AGG  
GCC CAT  
IgG1-Fos-3' CG TCT AGA TCA GGC GGC CAG GAT GAA CTC

PCR: 1) 2 min 94°C; 2) 2 min 59°C; 3) 2 min 70°C; 4) 29 x to 1; 5) 8 min 70°C

### Construction of fusion L8A4/IKappa and OKT3/Kappa sequences:

#### 1) L8A4-Kappa Primers

L8A4 5' LIGHT CGT TAA GCT TCA CCA TGA GTC CTG CCC AGT T  
L8A4/Kappa OL CAA GCT GGA AAT CAA ACG GAC TGT GGC TGC  
ACC ATCT  
Kappa 3' CGC CCT CGA GCT AAC ACT CTC CCC TGT TG

PCR: 1) 2 min 94°C; 2) 30 sec 94°C; 3) 1 min 62°C; 4) 1 min 72°C; 5) 2 x to 2; 6) 30 sec 94°C; 7) 1 min 58°C; 8) 1 min 72°C; 9) 4 x to 6; 10) 30 sec 94°C; 11) 1 min 65°C; 12) 1 min 72°C; 13) 21 x to 10; 14) 7 min 72°C

#### 2) OKT3-Kappa Primers

DKT3 5' LIGHT CCT CAA GCT TCA AAA TGG ATT TTC AAGTGCAG  
OKT3/Kappa OL CAA AGT TGG AAA TAA ACC GGA CTG TGG CTG  
CAC CAT CT  
Kappa 3' CGC CCT CGA GCT AAC ACT CTC CCC TGT TG

PCR: 1) 2 min 94°C; 2) 30 sec 94°C; 3) 1 min 58°C; 4) 1 min 72°C; 5) 2 x to 2; 6) 30 sec 94°C; 7) 1 min 50°C; 8) 1 min 72°C; 9) 4 x to 6; 10) 30 sec 94°C; 11) 1 min 60°C; 12) 1 min 72°C; 13) 21 x to 10; 14) 7 min 72°C

### Primers used for site-directed mutagenesis of L8A4-Jun:

IgG1-Jun OL2 CTC GAG CCG GGC GAT GCG CCC GCC TGC TGA  
GGA AGA GAT GGA GG  
IgG1-Jun OL3 CTT CCT CAG CAG GCG GGC GCA TCG CCC

PCR: 1) 30 sec 95°C; 2) 30 sec 95°C; 3) 1 min 55°C 4) 13.75 min 68°C; 5) 11 x to 2

### Primers used for Adaptor Cloning:

BsmAI-ApaI 5' TCA GCC GCC TCC ACC AAG GGC C  
BsmAI-ApaI 3' CTT GGT GGA GGC GG

### Primers used for sequencing reactions:

T7 Promoter Primer TAA TAC GAC TCA CTA TAG G  
BGH Reverse Primer TAG AAG GCA CAG TCG AGG

**Figure 2.3: Bispecific antibody construct sequences.** The OKT3 and L8A4 variable region sequences were reverse transcribed and amplified from RNA templates derived from the OKT3 and L8A4-producing hybridoma cell lines. The light chain kappa constant region was amplified from the pSV184 $\Delta$ Hneo-HuK template, and the heavy chain IgG1-derived sequence was amplified from the pSV2 $\Delta$ Hgpt-HuG1 template. Fos and Jun DNA templates were generated by PCR using four overlapping primers, as previously described (Kostelny, 1992). Variable region sequences are shown in bold type, as are the 6 base pair antibody-leucine zipper fusion junctions. Sequences representing sites for restriction enzyme digestion, designed into the primers for cloning purposes, are underlined. Different regions of heavy chain sequences are separated by forward slashes as follows: EcoRI restriction enzyme digestion site and kozac sequence///heavy chain variable region///beginning of human IgG1 constant region including CH1, hinge, and first codon of CH2 regions///antibody-leucine zipper fusion junction, encoding two glycines for increased junctional flexibility///fos or jun leucine zipper sequence///XbaI restriction enzyme digestion site. Different regions of light chain sequences are separated by forward slashes as follows: HindIII restriction enzyme digestion site and kozac sequence///light chain variable V-region///light chain variable J-region///human kappa sequence///XhoI restriction enzyme digestion site.

**OKT3-Fos sequence:**

GGAATTCACC//ATGGAAAGGCACTGGATCTTTCTACTCCTGTTGTCAGT  
AACTGCAGGTGTCCACTCCCAGGTCCAGCTGCAGCAGTCTGGGGCTG  
AACTGGCAAGACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCT  
GGCTACACCTTTACTAGGTACACGATGCACTGGGTAAAAACAGAGGCC  
TGGACAGGGTCTGGAATGGATTGGATACATTAATCCTAGCCGTGGTT  
ATACTAATTACAATCAGAAGTTCAAGGACAAGGCCACATTGACTACAG  
ACAAATCCTCCAGCACAGCCTACATGCAACTGAGCAGCCTGACATCTG  
AGGACTCTGCAGTCTATTACTGTGCAAGATATTATGATGATCATTACT  
GCCTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAG//CC  
TCCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCCTCCTCCAAGAGCACCTCT  
GGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG  
TGACGGTGTCTGTGGAATCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCCG  
GCTGTCTACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCC  
CTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCA  
GCAACACCAAGGTGGACAAGAAAGTTGGTGAGAGGCCAGCACAGGGAGGGA  
GGGTGTCTGCTGGAAGCCAGGCTCAGCGCTCCTGCCTGGACGCATCCCGGCTA  
TGCAGCCCCAGTCCAGGGCAGCAAGGCAGGCCCGCTCTGCCTCTTACCCGGA  
GGCCTCTGCCCCGCCCCACTCATGCTCAGGGAGAGGGTCTTCTGGCTTTTCCCC  
AGGCTCTGGGCAGGCACAGGCTAGGTGCCCCTAACCCAGGCCCTGCACACAA  
AGGGGCAGGTGCTGGGCTCAGACCTGCCAAGAGCCATATCCGGGAGGACCCT  
GCCCTGACCTAAGCCCCACCCCAAAGGCCAACTCTCCACTCCCTCAGCTCGG  
ACACCTTCTCTCCTCCCAGATTCCAGTAACTCCCAATCTTCTCTCTGCAGAGCCC  
AAATCTTGTGACAAAATCACACATGCCACCGTGCCAGGTAAGCCAGCCCA  
GGCCTCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAGTAGCCTGCATCC  
AGGGACAGGCCCCAGCCGGGTGCTGACACGTCCACCTCCATCTCTTCTCAGC  
A//GGCGGG//TTAACTGATACACTCCAAGCGGAGACCGACCAGCTGGAAGATA  
AGAAGTCTGCTCTGCAGACCGAGATTGCCAACCTGCTGAAGGAGAAGGAAAAA  
CTGGAGTTCATCCTGGCCGCCTGA//TCTAGACG

**OKT3-Kappa sequence:**

CCTCAAGCTTCAAA//ATGGATTTTCAAGTGCAGATTTTCAGCTTCCTGCT  
AATCAGTGCCTCAGTCATAATATCCAGAGGACAAATTGTTCTCACCCA  
GTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATGA  
CCTGCAGTGCCAGCTCAAGTGTAAGTTACATGAACTGGTACCAGCAG  
AAGTCAGGCACCTCCCCCAAAGATGGATTTATGACACATCCAACTG  
GCTTCTGGAGTCCCTGCTCACTTCAGGGGCAGTGGGTCTGGGACCTC  
TACTCTCTCACAATCAGCGGCATGGAGGCTGAAGATGCTGCCACTTA  
TACTGCCAGCAGTGGAGTAGTAACCCA//TTCACGTTCCGGCTCGGGGACA  
AAGTTGGAAATAAACCGG//ACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCC  
ATCTGATGAGCAGTTGAAATCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAA  
CTTCTATCCCAGAGAGGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAAT  
CGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTA  
CAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAG  
TCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGC  
TTCAACAGGGGAGAGTGTTAG//CTCGAGGGCG



**L8A4-Jun sequence:**

GGAATTCACC///ATGGGATGGAGCTGGATCTTTCTCTTTCTCCTGTCAGG  
AACTGCAGGTGTCCTCTCTGAGGTCCAGCTGCAACAGTCTGGACCTG  
AGCTGCTGAAGCCTGGGGCTTCAGTGAAGATATCCTGCAAGACTTCT  
GGATACACATTCACTGAATACACCATACTGGGTGAAGCAGAGCCA  
TGGAAAGAGCCTTGAGTGGATTGGAGGTATTGATCCTAACAATGGTG  
GTACTATGTACAACCAAAAATTCAAGGGCAAGGCCACATTGACTGTAG  
ACAAGTCTTCCAGCACAGCCTACACGGACCTCCGCAGCCTGACGTCT  
GAGGATTCTGCAGTCTATTACTGTACAAGAGCAGAGGCTATGGACTA  
CTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCGCCTCCACCA  
AGGGCC///CATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGG  
GCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGAC  
GGTGTCTGTGGAACCTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGT  
GTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCC  
AGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAA  
CACCAAGGTGGACAAGAAAGTTGGTGAGAGGCCAGCACAGGGAGGGAGGGT  
GTCTGCTGGAAGCCAGGCTCAGCGCTCCTGCCTGGACGCATCCCGGTATGCA  
GCCCCAGTCCAGGGCAGCAAGGCAGGCCCGTCTGCCTCTTACCCGGAGGC  
CTCTGCCCCGCCCACTCATGCTCAGGGAGAGGGTCTTCTGGCTTTTTCCCCAGG  
CTCTGGGCAGGCACAGGCTAGGTGCCCTAACCAGGCCCTGCACACAAAGG  
GGCAGGTGCTGGGCTCAGACCTGCCAAGAGCCATATCCGGGAGGACCCTGCC  
CCTGACCTAAGCCCACCCCAAAGGCCAACTCTCCACTCCCTCAGCTCGGACAC  
CTTCTCTCCTCCCAGATTCCAGTAACTCCCAATCTTCTCTCTGCAGAGCCCAAAT  
CTTGTGACAAAACCTCACACATGCCACCGTGCCAGGTAAGCCAGCCCAGGCC  
TCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAGTAGCCTGCATCCAGG  
GACAGGCCCCAGCCGGGTGCTGACACGTCCACCTCCATCTTCTCCTCAGCA///G  
GCGGG///CGCATCGCCCGGCTCGAGGAAAAAGTGAAAACCTTGAAAGCTCAGA  
ACTCGGAGCTCGCGTCCACGGCCAACATGCTCAGGGAACAGGTGGCACAGCTT  
AAACAGAAAGTCATGACTGA///TCTAGACG

**L8A4-Kappa sequence:**

CGTTAAGCTTCACC///ATGAGTCCTGCCCAGTTCCTGTTTCTGTAGTGCT  
CTGGATTCAAGGAAACCAACGGTGATGTTGTGATGACCCAGACTCCAC  
TCACTTTGTGCGTTACCATTGGACAACCAGCCTCTATCTCTTGCAAGT  
CAAGTCAGAGCCTCTTATATAGTAATGGAAAAACCTATTTGAATTGGT  
TATTACAGAGGCCAGGCCAGTCTCCAAAGCGCCTAATCTATCTGGTAT  
CTAAACTGGACTCTGGAGTCCCTGACAGGTTCACTGGCAGTGGATCA  
GGAACAGATTTTACACTGAAAATCAGCAGAGTGGAGGCTGAGGATTT  
GGGAATTTATTACTGCGTGCAAGATACACATTTTCCT///CAGACATTCGGT  
GGAGGCACCAAGCTGGAAATCAAACGG///ACTGTGGCTGCACCATCTGTCTTCA  
TCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAGTGCCTCTGTTGTGTGCC  
TGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAAC  
GCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGCAAGG  
ACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGAG  
AAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGT  
CACAAAGAGCTTCAACAGGGGAGAGTGTTAG///CTCGAGGGCG

Reaction with Modifying Enzymes: Restriction endonucleases (New England Biolabs, Beverly MA, or Gibco BRL), calf intestinal alkaline phosphatase (CIP) (New England BioLabs), DNA Polymerase I large fragment (Klenow) (New England BioLabs), and T4 DNA ligase (New England BioLabs) were used according to the manufacturer's instructions.

Bacterial Transformation/Isolation of Plasmid DNA: The following bacteria were used for transformation with plasmid DNA: DH5 $\alpha$  competent (Gibco BRL); DM1 competent (Gibco BRL); INV $\alpha$ F' competent (One Shot Kit, Invitrogen); XL1-Blue supercompetent (QuikChange™ Site-Directed Mutagenesis Kit, Stratagene); and XL10-Gold ultracompetent (Stratagene). Each bacterial cell type was transformed according to the manufacturer's instructions. Plasmid DNA was isolated using the Wizard *Plus* Minipreps DNA Purification System (Promega) or the QIAfilter Plasmid Maxi Kit (Quiagen), according to the manufacturer's instructions.

Adaptor Cloning: The adaptor cloning method was used to correct a portion of the L8A4-Jun bispecific antibody construct which contained an extraneous sequence (see Figure 4.6). Synthetic complementary oligonucleotides encoding the corrected sequence, which formed double stranded DNA upon annealing of the two primers, were chemically synthesized by Gibco BRL. This double stranded "adaptor" was engineered to end with sticky ends, complementary on each side to those produced by digestion of the original construct with BsmAI and ApaI restriction enzyme sites flanking the extraneous sequence. The double stranded adaptor was generated from the two complementary primers as follows: 1  $\mu$ L of 5' and 3' adaptor oligonucleotides (1  $\mu$ g/ $\mu$ L) were combined individually with 2  $\mu$ L of 10 mM ATP, 2  $\mu$ L 10X kinase buffer, 14  $\mu$ L LoTE (3 mM Tris, 0.2 mM EDTA, pH 7.5), and 1  $\mu$ L T<sub>4</sub> kinase. The mixtures were incubated for 30

minutes at 37°C, followed by heat inactivation at 65°C for 20 minutes. The oligonucleotide mixtures were then combined and 10 µL 5X T<sub>4</sub> ligase buffer was added. The samples were boiled for 2 minutes, slowly cooled to room temperature, and stored at -20°C until use. The adaptor cloning ligation reaction was performed at molar ratios of 2:1 to 20:1, adaptor:vector.

**Site Directed Mutagenesis:** The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA) was used according to the manufacturer's instructions. Briefly, synthetic complementary oligonucleotide primers containing the desired base pair change were chemically synthesized by Gibco BRL. 10 ng of double stranded DNA template was combined with 125 ng of each primer, 5 µL of 10X Pfu reaction buffer, 1 µL of 10 mM dNTP mix, and 1 µL Pfu DNA polymerase (2.5 U/µL) in a total volume of 50 µL. The reaction was heated to 95°C for 30 seconds, followed by 12 cycles of the following: 1) 95°C x 30 seconds, 2) 55°C x 60 seconds, 3) 68°C x 13 minutes, 45 seconds. The bacterial DNA templates were then nicked by incubation with 1 µL of DpnI (10 U/µL) to digest parental non-mutated bacterial DNA. PCR-generated mutagenized vectors were transformed into XL-1 Blue Supercompetent cells and screened for the desired mutation by restriction enzyme digestion and sequencing of vector DNA (see Figure 4.7).

**DNA Sequencing:** Manual sequencing was performed using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland OH), according to the manufacturer's instructions. Automated sequencing of the final bsAb constructs was performed by the Duke University Sequencing Facility.

**Chapter 3**

**BRCA2 Monoclonal Antibodies  
React with  
Differentiating Epithelium**

## Introduction

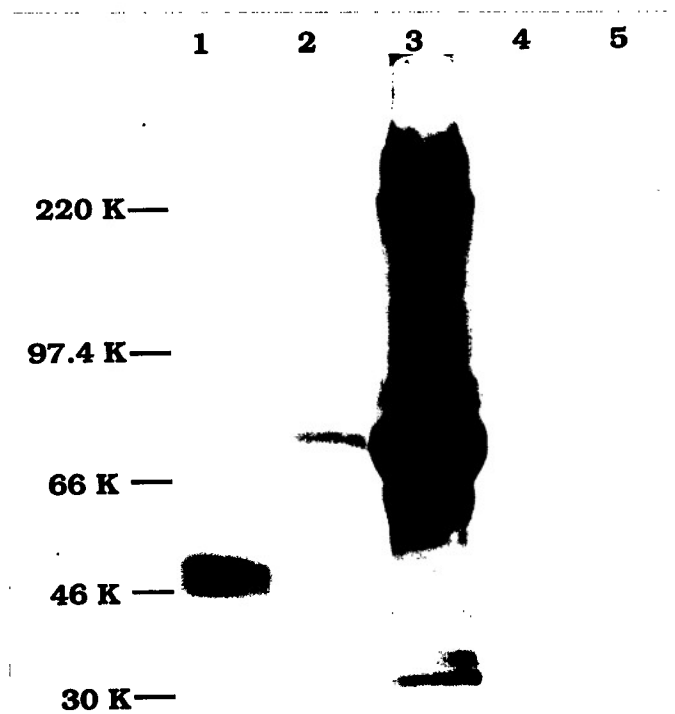
Individuals with germline mutations in the BRCA2 breast cancer susceptibility gene are at increased risk for breast cancer (including male breast cancer), ovarian cancer, pancreatic cancer, and prostate cancer. Very little is known about either the normal cellular functions of BRCA2 or how its mutation predisposes to malignancy. Recently, BRCA2 protein has been immunoprecipitated from nuclear fractions of breast and bladder carcinoma cells, and its expression was shown to be upregulated with the cell cycle, with induction at late G<sub>1</sub>/early S phase (Bertwistle, 1997; Chen, 1998). However, no descriptions of the normal cellular and tissue distribution of this protein have been published to date. Here we report the distribution of BRCA2 protein expression using novel monoclonal and polyclonal antisera. We have found immunohistochemical reactivity of BRCA2 antibodies with differentiated normal epithelia of skin and thymus, as well as in squamous cell carcinoma of head and neck origin, but not in normal or malignant breast epithelium. In addition, we have found anti-BRCA2 reactivity in several human fetal tissues, with staining localized to cells undergoing differentiation. This distribution has implications for the function of the BRCA2 gene product. In addition to its proposed role in proliferative cells as a molecule important in monitoring the integrity of DNA, BRCA2 may play also an important as yet undefined, role in the process of differentiation.

## Results

### **MAb 2C9 Specifically Recognizes BRCA2 Protein.**

We first developed an anti-BRCA2 monoclonal antibody called 2C9 using the 49 kDa BAC2 peptide as an immunogen (see Chapter 2). To determine the specificity of mAb 2C9, we first performed Western blot analysis. 2C9 was strongly reactive against the 49 kDa purified BAC2 immunogen, but was not reactive against the non-overlapping purified BAC1 (Figure 3.1). In addition, when bacterial lysates containing BAC2 peptide fused to glutathione-S-transferase (GST) were blotted, 2C9 detected a shift in molecular weight to 79 kDa, further confirming the BAC2-specificity of 2C9. 2C9 was not reactive against protein from similar bacterial lysates containing the BAC1-GST fusion protein, or from those producing GST alone (not shown).

To further define the BRCA2 epitope recognized by mAb 2C9, we prepared lysates from bacterial or yeast cells transformed with various BRCA2 expression constructs (Figure 3.2). These studies localized the reactivity of mAb 2C9 to aa 1264-1329, corresponding to bp 4021-4215 of BRCA2 exon 11. In addition, mAb 2C9 reacted with a band consistent with the predicted 384 kDa MW of BRCA2 in Cos-7 cells transfected with full length BRCA2 cDNA (pBSX/BRCA2) by Western blot. The band is identical to that detected by polyclonal rabbit antiserum #5815, raised against non-overlapping sequences of BRCA2. Isotype-matched negative control mAb P3x63 and normal rabbit serum do not detect bands at this molecular weight (Figure 3.3).



**Figure 3.1: MAb 2C9 reacts with immunizing peptide BAC2 and its fusion protein BAC2-GST.** Lysates were prepared from uninduced (lane 2) and induced (lane 3) bacteria producing the BAC2-GST fusion protein (BRCA2 bp 3748-4816) as well as from uninduced (lane 4) and induced (lane 5) bacteria producing the BAC1-GST fusion protein (BRCA2 bp 2716-3714). The lysates and GST-cleaved and purified BAC2 protein were separated on a 7.5% polyacrylamide gel. 2C9 was reactive with the purified BAC2 protein at the expected MW of 49 kDa (lane 1) as well as with the 79 kDa BAC2-GST fusion protein (lanes 2, 3). 2C9 was non-reactive with all components of the lysates from BAC1-GST producing bacteria (lanes 4, 5).

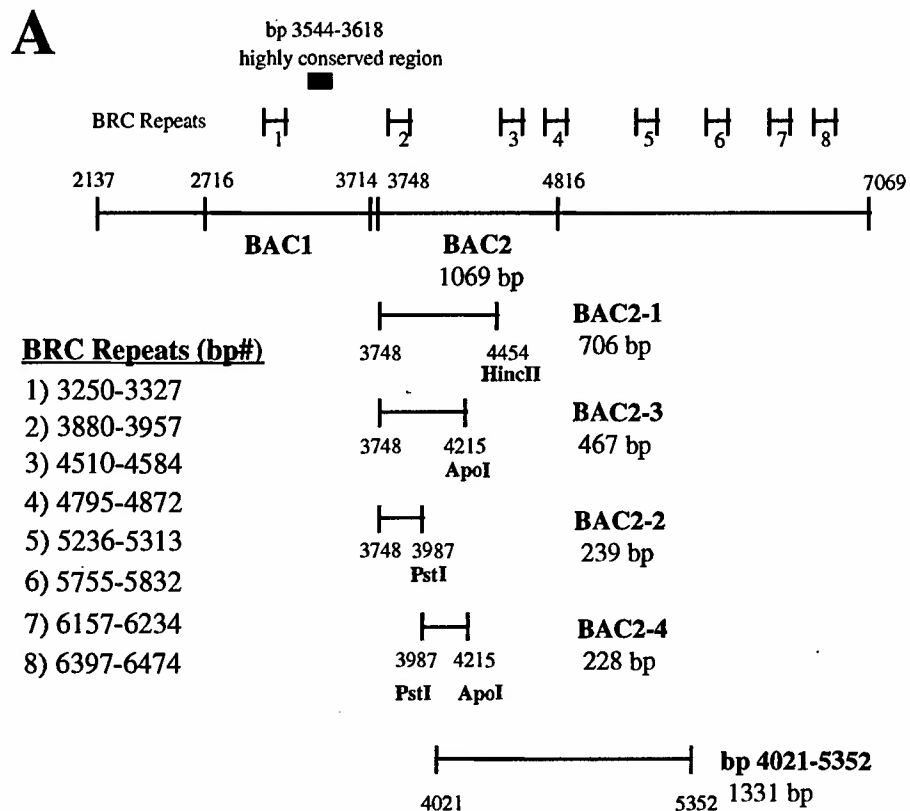
**Figure 3.2: Reactivity of mAb 2C9 with BRCA2 exon 11 constructs.**

A) The BRCA2 peptides studied are shown on this diagram of exon 11, along with structural features of this region of the BRCA2 gene, including BRC-repeats #1-8 (Bork, 1996) and a region which is highly conserved among different species (Bignell, 1997).

C) Induced lysates from bacteria or yeast transformed with the indicated BRCA2 sequences were analyzed on 10% SDS-PAGE gels, transferred to nitrocellulose, and reacted with 2C9 in a Western blot assay. 2C9 was reactive with bacterial proteins containing the immunizing peptide BAC2, bp 3748-4816 (lane 1); deletion constructs BAC2-1, bp 3748-4454 (lane 2); BAC2-3, bp 3748-4215 (lane 3); BAC2-4, bp 3987-4215 (lane 4); and yeast-derived BRCA2 sequences, bp 4021-5352 (not shown); but not with deletion construct BAC2-2, bp 3748-3987 (lane 5) or BAC1, bp 2716-3714 (Figure 3.1), derived from a different portion of BRCA2 exon 11. These studies define the 2C9 epitope to be contained within BRCA2 aa 1264-1329, encoded by bp 4021-4215.

Reactivity of BRCA2 deletion constructs with 2C9 is summarized in panel B. The yeast bp 4021-5352 construct was produced and tested by Dr. Aurora Pryor in the laboratory of Dr. Jeffrey Marks.



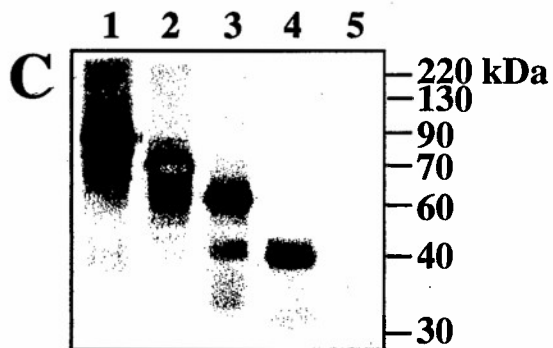


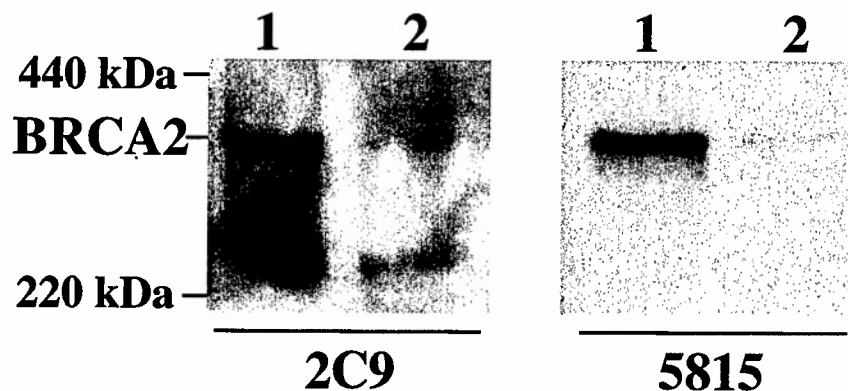
**BRC Repeats (bp#)**

- 1) 3250-3327
- 2) 3880-3957
- 3) 4510-4584
- 4) 4795-4872
- 5) 5236-5313
- 6) 5755-5832
- 7) 6157-6234
- 8) 6397-6474

**B Reactivity with 2C9**

Full length	+
BAC1	-
BAC2	+
BAC2-1	+
BAC2-3	+
BAC2-2	-
BAC2-4	+
bp 4021-5352	+





**Figure 3.3: BRCA2 antibodies recognize full length BRCA2 in Cos-7 cells.** Lysates from Cos-7 cells transfected with pBSX/BRCA2 (lane 1) or mock transfected (lane 2) were reacted with mAb 2C9 (left panel) or polyclonal rabbit serum #5815 (right panel) in Western blot assays. Identical bands consistent with the 384 kDa predicted molecular weight of BRCA2 were detected by both 2C9 and #5815. Extremely faint bands at the same molecular weight are also seen in lysates from mock transfected Cos-7 cells. As African green monkey BRCA2 is 94% identical (61 of 65 aa) with human BRCA2 in the 2C9 epitope region (aa 1264 to 1329) and 90% identical (304 of 338 aa) with human BRCA2 in the BAC1 region (aa 829 to 1162), this most likely represents detection of endogenous Cos BRCA2. No bands at this molecular weight were detected using control mAb P3x63 or normal rabbit serum.

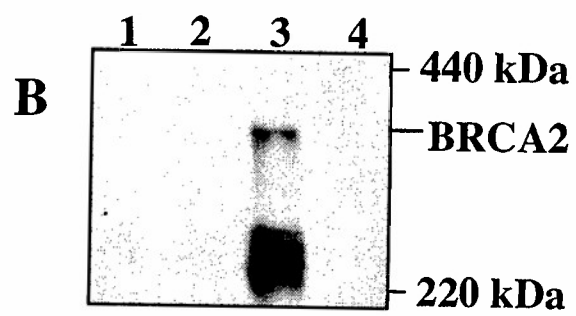
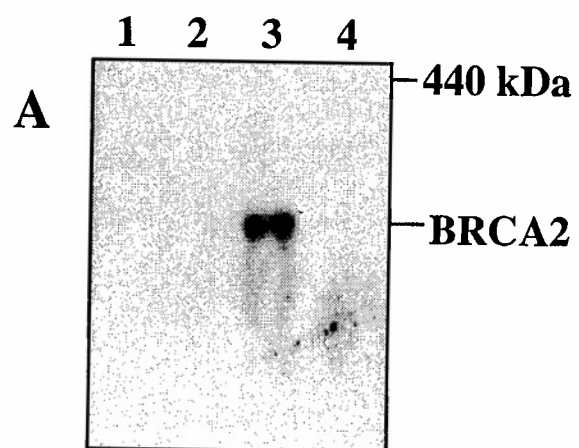
### **BRCA2 is a High Molecular Weight Protein Expressed at Very Low Levels in Breast and Ovarian Carcinoma Cells.**

No reactivity above background was demonstrable using mAb 2C9 on frozen sections of breast carcinoma or normal breast tissue by immunohistochemistry, even when using the extremely sensitive immunoenhancing technique of catalyzed signal reporter deposition (Kerstens, 1995). Similarly, BRCA2 protein was not detected using 2C9 in standard Western blot assays or flow cytometry of MCF-7 breast carcinoma cells, previously shown to express BRCA2 mRNA under conditions of serum-starvation and release (Vaughn, 1996). These studies suggested that BRCA2 protein may be present at very low levels within cells, requiring the use of antigen concentration techniques for detection. Therefore we used 2C9 to immunoprecipitate (and thus concentrate) BRCA2 protein from lysates of MCF-7 breast carcinoma cells, followed by Western blot detection with anti-BRCA2 immunological reagents (polyclonal anti-BRCA2 mouse serum or mAb 2C9). These studies demonstrated specific immunoprecipitation of high molecular weight bands consistent with the predicted size of 384 kDa of the BRCA2 protein (Figure 3.4). Identical results were seen in experiments performed by Ed Lobenhofer in the laboratory of Dr. Jeffrey Marks, using protein lysates from MCF-7, BT483, and T47D breast cancer cell lines as well as SKOV3 ovarian cancer cells.

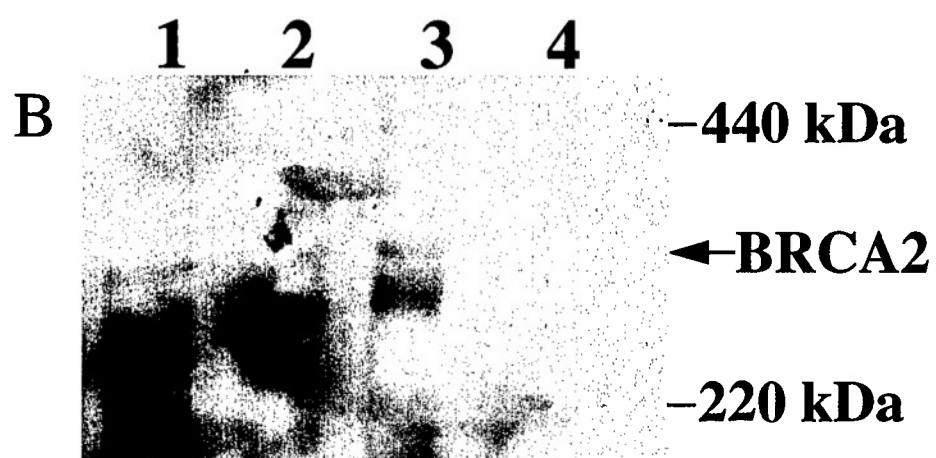
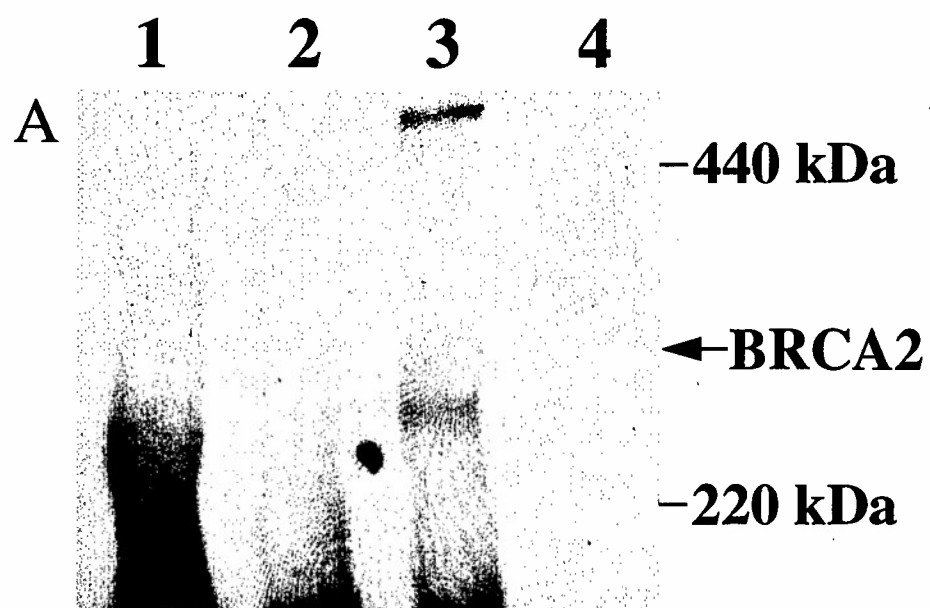
### **BRCA2 Protein is Tyrosine Phosphorylated.**

Since the levels of BRCA2 message have previously been shown to be regulated by the cell cycle, and because the cellular levels of cell cycle-associated proteins are often regulated by phosphorylation, we next analyzed cellular BRCA2 proteins for tyrosine phosphorylation using immunoprecipitation/ Western blot assays. Either 2C9 or anti-phosphotyrosine mAb 2G8.D6 was used to precipitate BRCA2 protein from Cos-7 cells

**Figure 3.4: MAb 2C9 immunoprecipitates a high molecular weight protein consistent with BRCA2 from breast carcinoma cells.** MCF-7 cells were serum starved for 3 days, then released from starvation for 1 day, followed by lysis and protein extraction. The lysate from  $17 \times 10^6$  cells (panel A) or  $56 \times 10^6$  cells (panel B) was immunoprecipitated with mAb 2C9 as described. Immunoprecipitates were separated on a 4% SDS polyacrylamide gel, and Western blot analysis was performed. Bands were detected with either polyclonal anti-BAC2 serum (panel A) or with 2C9 (panel B). A band consistent with the 384 kDa predicted MW of BRCA2 protein is specifically immunoprecipitated by 2C9 in both experiments (lane 3). No specific reactivity is seen with proteins immunoprecipitated by negative control antibody (preclear #1-lane 1; preclear #2-lane 2), or with proteins remaining in the lysate following 2C9 immunoprecipitation (lane 4). In panel B, lower molecular weight bands likely correspond to products of mRNA splice variants, differentially processed protein, or protein degradation products. These lower molecular weight species were also seen from lysates of other breast and ovarian carcinoma cell lines immunoprecipitated with 2C9, in experiments performed by Ed Lobenhofer in the laboratory of Dr. Jeffrey Marks (not shown). In panel A, in order to ensure maximal separation of high molecular weight species, the 220 kDa molecular weight marker was run off the gel; lower molecular weight bands, as seen in other experiments would likely have been run off as well.



**Figure 3.5: BRCA2 protein is tyrosine phosphorylated.** We performed cross-immunoprecipitation/Western blot assays on Cos-7 cells transfected with pBSX/BRCA2, as described. Protein extracts from  $5 \times 10^6$  cells were immunoprecipitated twice with negative control mAb P3x63 (preclear #1 - lane 1; preclear #2 - lane 2), followed by immunoprecipitation with specific mAbs 2C9 (panel A, lane 3) or anti-phosphotyrosine mAb 2G8.D6 (panel B, lane 3). Incubation of the blots with either mAb 2G8.D6 (panel A) or mAb 2C9 (panel B) revealed identical high molecular weight bands in each lane representing an immunoprecipitation using the other specific antibody (lane 3). No reactivity is seen with proteins immunoprecipitated in the preclear steps (lanes 1 and 2), or with proteins remaining in the lysate following immunoprecipitation (lane 4). In addition to a high molecular weight band, lower molecular weight species were noted in both experiments. Implications for these findings are discussed in Figure 3.4.



transfected with the full length BRCA2 cDNA construct, with the other antibody being used in the detection procedure. Identical high molecular weight bands were seen in both cross precipitation experiments (Figure 3.5), suggesting that at least a fraction of the cellular BRCA2 protein is tyrosine phosphorylated. Similar results were seen in cross immunoprecipitation experiments performed by Ed Lobenhofer in the laboratory of Dr. Jeff Marks, using protein lysates from breast and ovarian cancer cell lines.

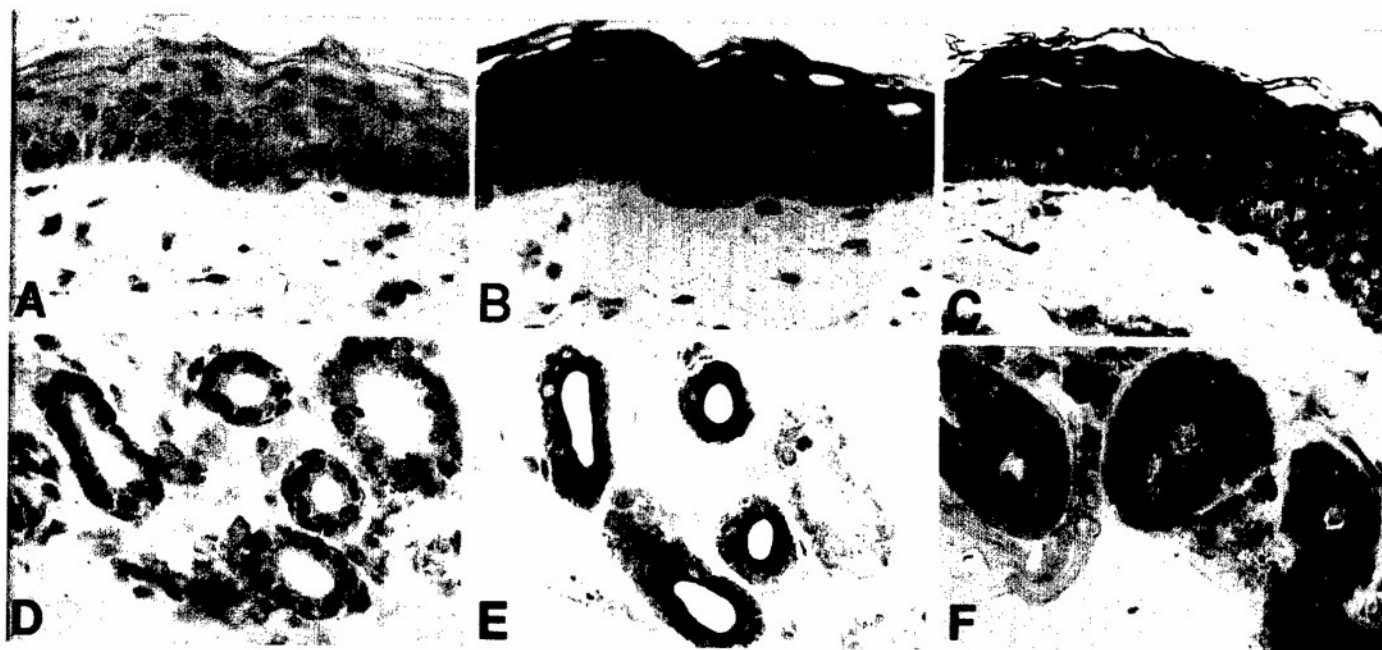
#### **BRCA2 mAb 2C9 is Reactive with Terminally Differentiated Normal Epithelial Tissues.**

Since mAb 2C9 was non-reactive with normal breast and breast carcinoma tissues, we next screened a panel of acetone-fixed frozen tissues, emphasizing tissues such as thymus, which have high expression of BRCA2 mRNA (Tavtigian, 1996). The tissues analyzed included skin, thymus, small and large intestine, appendix, liver, kidney, heart, skeletal muscle, tonsil, lymph node, and pancreas. The majority of these tissues were non-reactive with BRCA2 mAb 2C9, as seen for normal breast and breast cancer tissues. However, 2C9 mAb reacted strongly with terminally differentiated thymic epithelial cells present in the Hassall's bodies of normal pediatric thymus (Figure 3.6), as well as with the suprabasal layers of adult epidermis (Figure 3.7). Similarities in expression of a variety of antigens have previously been observed for thymic epithelial cells and epidermal keratinocytes, which is not surprising as both consist of epithelial cells continually undergoing terminal differentiation in post-natal life (Laster, 1986; Patel, 1995). Addition of the BAC2 peptide to tissue sections prior to the addition of mAb 2C9 blocked 2C9's reactivity (data not shown).





**Figure 3.6: BRCA2 mAb 2C9 is reactive with terminally differentiated epithelial cells in thymus.** In a comprehensive immunohistochemical screen of frozen acetone-fixed tissue sections to identify tissues with potentially higher levels of BRCA2 protein, we found strong cytoplasmic reactivity with 2C9 mAb (indicated by dark brown color) in the terminally differentiating thymic epithelial cells present in the Hassall's bodies of normal pediatric thymus (panel B). Panel A shows lack of similar reactivity with isotype-matched control mAb, P3X63. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.



**Figure 3.7: BRCA2 mAb 2C9 is reactive with terminally differentiated cells in adult human skin.** MAb 2C9 reacts with the suprabasal layers of adult epidermis (panel B) and with eccrine ducts in the dermis (panel E). Panels A and D show corresponding negative reactivity with isotype-matched control mAb P3X63. For comparison, the pattern of reactivity with mAb AE-2 (panels C, F), specific for the 56.5 and 65.5 kDa cytokeratins (CK 1, 2, and 10) characteristic of terminally differentiated epithelial cells (Moll, 1982; Woodcock-Mitchell, 1982), differs from that seen with mAb 2C9. MAb 2C9 reacts with eccrine ducts but not with the secretory portion of the eccrine coil (panel E), while AE-2 demonstrates significant reactivity with all portions of the eccrine gland (panel F). In addition, while 2C9 shows uniform reactivity with suprabasal layers of the epidermis (panel B), AE-2 shows granular staining, with increasing reactivity with distance above the basal epidermal layer (panel C). Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

To further determine the specificity of this pattern of reactivity, we then tested monoclonal antibodies directed against different portions of the BRCA2 molecule. MAb 5F6 (raised against a portion of exon 14, see Chapter 2) also reacts with Hassall's bodies in thymus and with epidermis (Figures 3.8 and 3.9), although 2 other monoclonal antibodies (3E6 and 9D3) as well as the rabbit polyclonal serum (#5815) were non-reactive (not shown). These differences in reactivity may be due to the availability of the particular epitopes against which each antibody is directed in these tissues, or to differences in Ab reactivity with native protein conformations. In addition, mAbs 2C9 (exon 11), 5F6 (exon 14), and 3E6 (exons 7-10) all show similar reactivity with cellular extracts from skin by Western blot analysis. (Figure 3.10)

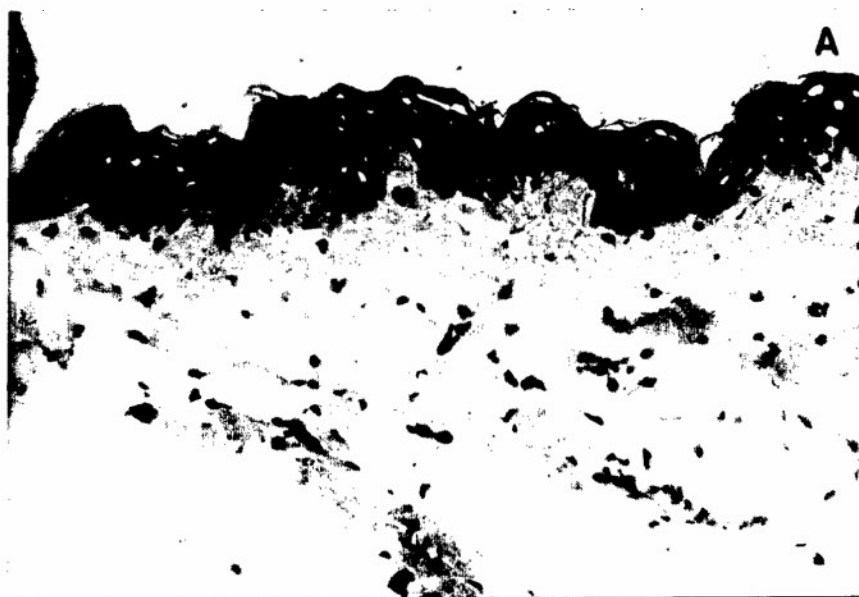
#### **BRCA2 mAb 2C9 is Reactive with Squamous Cell Carcinomas.**

The pattern of reactivity of 2C9 with differentiating epithelium suggested that BRCA2 protein may be stabilized or upregulated under conditions favoring differentiation of epithelium. Therefore, we next examined the reactivity of this antibody with a panel of moderate to well differentiated squamous cell carcinomas (SCCs) of head or neck origin. MAb 2C9 demonstrated strong reactivity with 9 of 10 formalin-fixed paraffin-embedded well differentiated SCCs (Figure 3.11). 2C9 was also reactive with 1 of 4 frozen, moderately differentiated SCCs. The frozen tumor which was reactive with mAb 2C9 also reacted with mAb 5F6, directed against a different portion of the BRCA2 molecule (Figure 3.12), suggesting that these antibodies are specifically reacting with BRCA2 in SCCs.

**Figure 3.8: BRCA2 mAbs 2C9 and 5F6 demonstrate similar immunohistochemical reactivity with terminally differentiated epithelial cells in thymus.** Similar reactivity of two anti-BRCA2 mAbs generated against different portions of the BRCA2 molecule was seen in terminally differentiating thymic epithelial cells present in the Hassall's bodies of normal pediatric thymus. Panel B shows positive reactivity with mAb 2C9, (exon 11), and panel C shows similar positive reactivity with mAb 5F6 (exon 14). Panel A shows reactivity with isotype-matched control mAb, P3X63. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

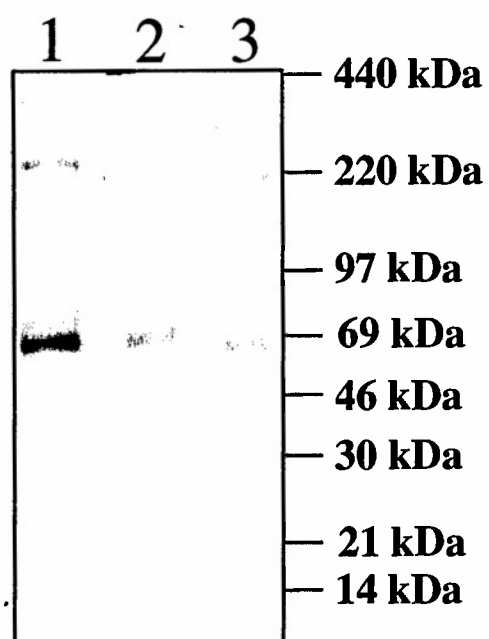


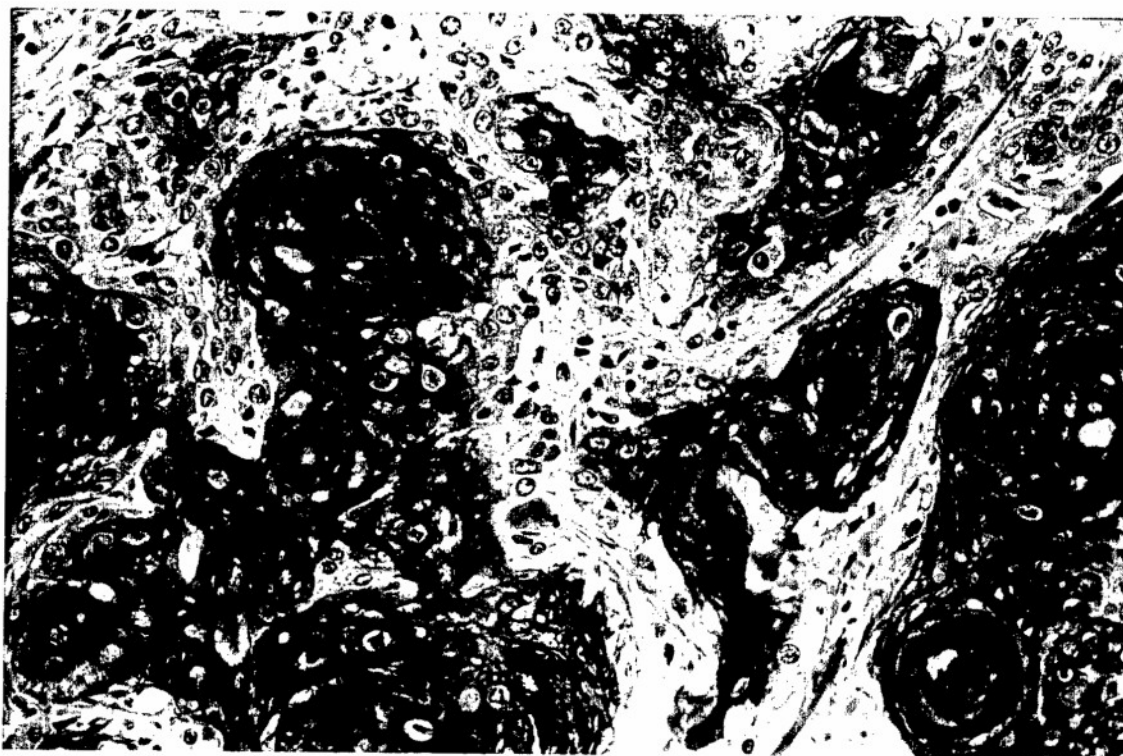
**Figure 3.9: BRCA2 mAbs 2C9 and 5F6 demonstrate similar immunohistochemical reactivity with terminally differentiated epithelial cells in adult human skin.** Two anti-BRCA2 mAbs generated against different portions of the BRCA2 molecule demonstrated similar reactivity with the suprabasal layers of adult epidermis. Panel A shows positive reactivity with mAb 2C9 (exon 11), and panel B shows similar positive reactivity with mAb 5F6 (exon 14). The reactivity observed was not as strong for mAb 5F6 as for mAb 2C9. This may be due to the accessibility of different epitopes in this tissue. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.



**Figure 3.10: BRCA2 mAbs 2C9 and 3E6 demonstrate similar reactivity with epidermis by Western blot analysis.** Human foreskin epidermis was isolated by trypsinization, and protein extracts were made using the urea extraction method described in Chapter 2. These extracts were analyzed on a 4-20% gradient gel, transferred to nitrocellulose, and reacted with mAbs 2C9 (lane 1), 5F6 (lane 2), and 3E6 (lane 3) in a Western blot assay. All three antibodies detected a stronger band at a molecular weight in the 50-60 kDa range, and a band with a molecular weight of approximately 220 kDa was detected by mAbs 2C9 and 3E6. No reactivity was seen with any of the three antibodies in this Western blot analysis at the higher molecular weight of 384 kDa predicted for full-length BRCA2. Negative control antibody P3x63 was not used in this experiment; however, in an experiment performed under identical conditions, a non-specific band in the 50-60 kDa range was detected by P3, suggesting that this band is probably non-specific (see Figure 3.17). However, no P3 reactivity was observed with any 220 kDa species in that experiment, indicating that the reactivity seen in this experiment of the mAbs 2C9 and 3E6 at 220 kDa is specific for BRCA2.

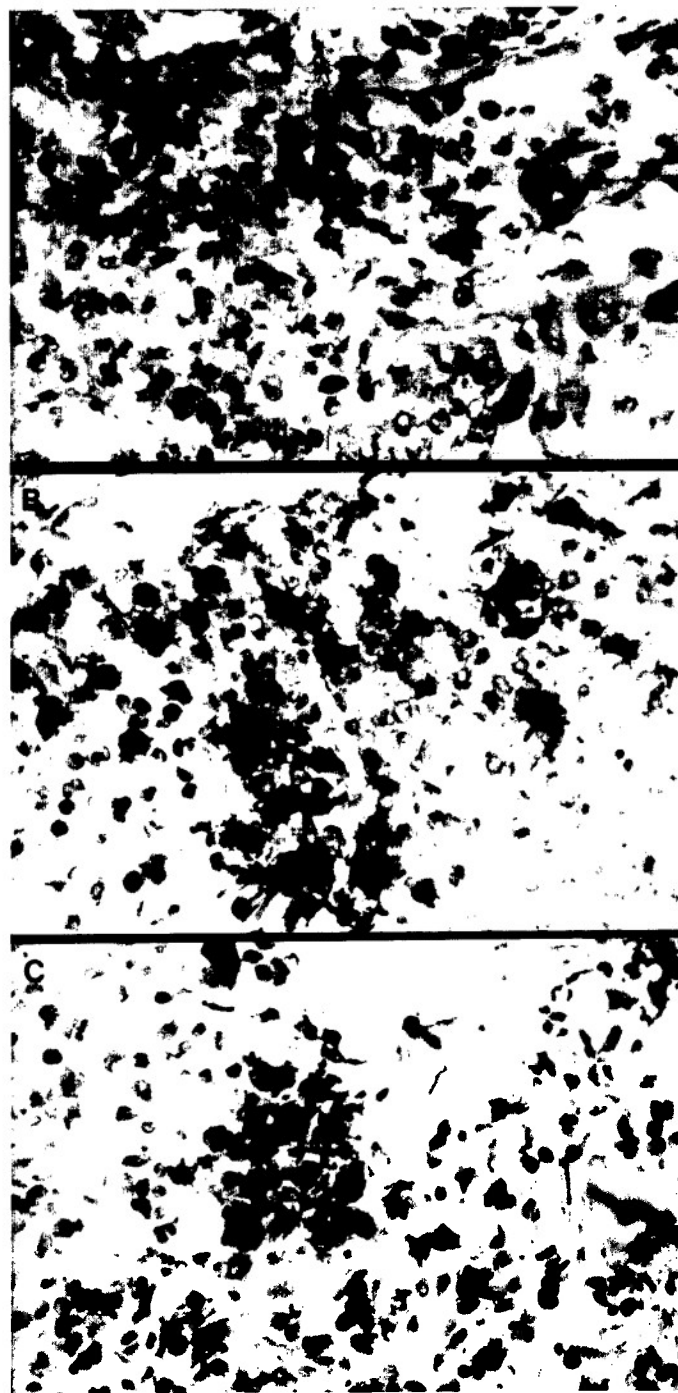






**Figure 3.11: BRCA2 mAb 2C9 reacts with squamous cell carcinomas.** MAb 2C9 demonstrated strong reactivity with 9/10 of well differentiated squamous cell carcinomas (SCCs) of head or neck origin. No reactivity was seen with isotype-matched negative control mAb P3X63 (not shown). Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

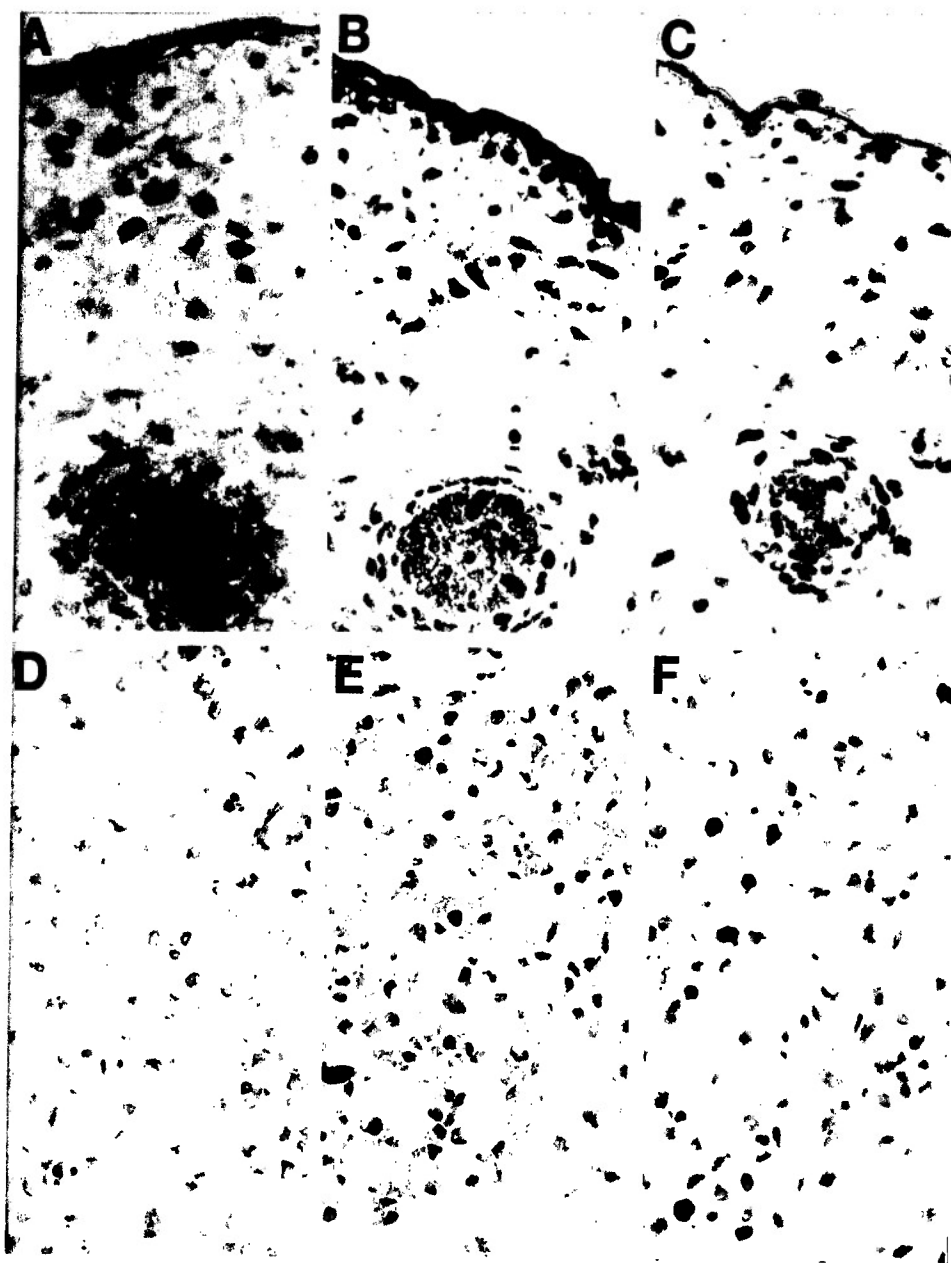
**Figure 3.12: BRCA2 mAbs 2C9 and 5F6 demonstrate similar immunohistochemical reactivity with squamous cell carcinomas.** Two anti-BRCA2 mAbs generated against different portions of the BRCA2 molecule demonstrated similar reactivity with squamous cell carcinomas. Panel B shows positive reactivity with mAb 2C9 (exon 11), and panel C shows similar positive reactivity with mAb 5F6 (exon 14). Panel A shows lack of reactivity with isotype-matched control mAb, P3X63. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.



### **BRCA2 mAb 2C9 is Reactive with Differentiating Fetal Tissues.**

To further investigate the role of BRCA2 protein in cellular differentiation, we studied reactivity of 2C9 mAb with a panel of 16 week human fetal tissues, in which a much higher fraction of cells in a given tissue are actively involved in the differentiation process. In contrast to the limited tissue distribution of mAb 2C9 immunohistochemical reactivity in adults, mAb 2C9 demonstrated reactivity with a wider range of fetal tissues, including skin, skeletal muscle, smooth muscle, cardiac muscle, and possibly gut, in a subset of epithelial cells (Figure 3.13). Strong reactivity was associated with differentiation, and not with cellular proliferation. Cells expressing the highest levels of protein reactive with this anti-BRCA2 antibody included cells of the periderm (the fetal equivalent of the upper, more differentiated layers of the adult epidermis) and differentiating myoblasts. In addition, 2C9 reactivity was noted in the goblet cells in the fetal gut, the most differentiated epithelial cells in this tissue, although it was not clear whether low level reactivity in these cells might be artifactually enhanced by compression of the cytoplasm by enclosed mucin. Similar to results in adult tissues, cells reactive with BRCA2 mAb 2C9 were non-reactive with the mib-1 mAb specific for the Ki-67 nuclear antigen expressed in proliferating cells, implying that BRCA2 might have some function in non-proliferative cells, in addition to the previously hypothesized functions in cycling cells.

**Figure 3.13: BRCA2 mAb 2C9 reacts with terminally differentiating epithelial and muscle cells in fetal tissue.** Immunohistochemical analysis of a panel of 16 week human fetal tissues revealed strong 2C9 reactivity with the periderm (the fetal analog of the upper layers of the adult epidermis) but not with the germinal layer of fetal skin (panel B). In addition, faint but significant reactivity was observed on differentiating skeletal muscle (panel E). Cells reactive with BRCA2 mAb 2C9 were non-reactive with the mib-1 mAb specific for the Ki-67 nuclear antigen expressed in proliferating cells (panels C, F). Panels A and D show negative reactivity with isotype-matched control mAb P3x63. Similar results were seen in 12 week fetal tissues. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.



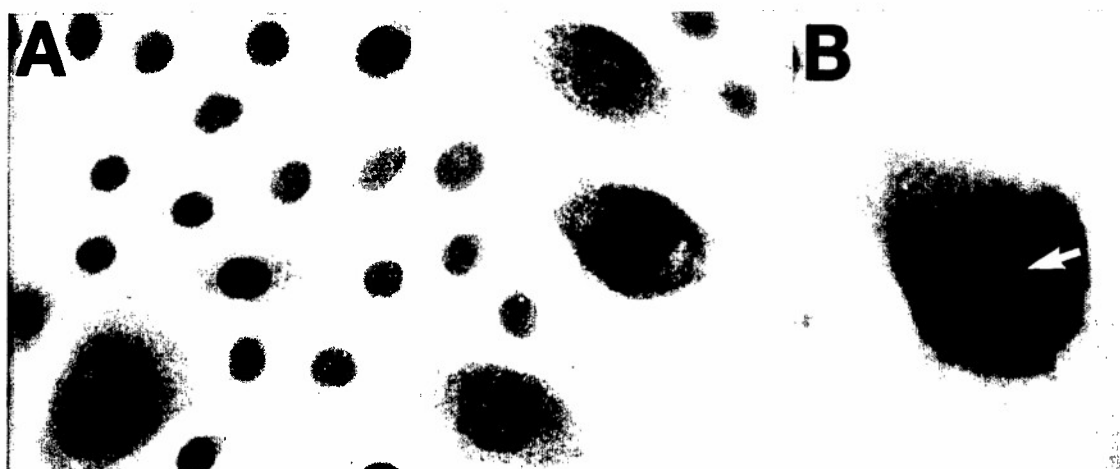
### **BRCA2 mAb 2C9 Immunoreactivity Increases in Differentiating Keratinocytes.**

We further confirmed mAb 2C9 reactivity with differentiating epithelium using immunoperoxidase assays on epidermal keratinocytes cultured *in situ* onto glass slides. Keratinocytes cultured in media containing either increased calcium concentrations (1.2 mM) or fetal calf serum (FCS) undergo characteristic changes associated with differentiation, including changes to squamous morphology and upregulation of molecules that are considered classical markers of differentiation, including high molecular weight cytokeratins 1, 2, and 10, filaggrin, loricrin, and transglutaminase. Little 2C9 immunoreactivity is seen in epidermal keratinocytes at baseline ( $t=0$ ). Reactivity is limited to a few scattered cells which are larger, more spread out (squamous), and more differentiated-appearing than cells which are non-reactive with 2C9 (Figure 3.14A). Increased 2C9 immunoreactivity is noted after several hours of incubation in differentiation medium, and 2C9 immunostaining remains strongest in the large, squamoid, more differentiated cells (which are much more numerous after several hours of differentiation). The staining pattern in these cells is cytoplasmic with perinuclear accentuation and nuclear dots (Figure 3.14B).

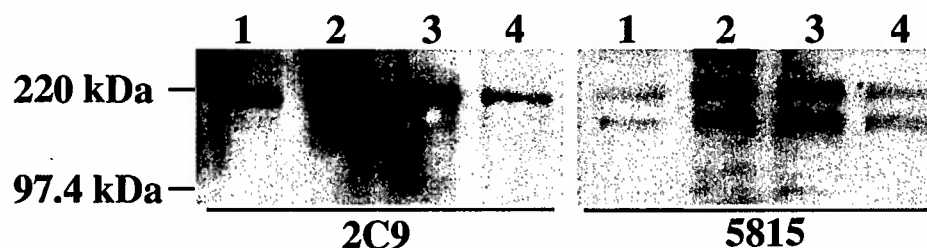
### **Proteins Reactive with mAb 2C9 and Polyclonal Anti-BRCA2 Antibodies Increase with Differentiation of Keratinocytes.**

Western blot analysis of extracts from cultured epidermal keratinocytes revealed mAb 2C9 reactivity with a high molecular weight band that transiently increased in intensity after induction of differentiation. This band was identical to that detected by anti-BRCA2 rabbit polyclonal serum #5815, directed against a non-overlapping portion of exon 11 (Figure 3.15). Although this band was detected at a lower molecular weight (<220 kDa) than expected for the predicted 384 kDa BRCA2 protein, it was specifically detected by two antibodies directed against different portions of the BRCA2 molecule,





**Figure 3.14: BRCA2 mAb 2C9 reactivity in cultured epidermal keratinocytes.** 2C9 reactivity was localized using immunoperoxidase assays on epidermal keratinocytes derived from neonatal foreskins and cultured *in situ* onto glass slides. 2C9 immunoreactivity was cytoplasmic and in many cells, appeared to be perinuclear, with a thin rim of unstained cytoplasm present between the nucleus and the greatest intensity of staining. Nuclear dots were notable at high magnification (panel B, arrow). Reactivity was strongest on larger, more squamous cells. Similar results were obtained using immunofluorescence staining. The overall staining intensity of cultured keratinocytes is much lower than that seen in tissue sections of epidermis, suggesting that factors which up-regulate expression of BRCA2 protein *in vivo* are not present in our keratinocyte cultures. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.



**Figure 3.15: Proteins reactive with BRCA2 antibodies transiently increase with differentiation of keratinocytes.** Human foreskin keratinocytes cultured in differentiation (high calcium) medium were harvested at 0 (lane 1), 4 (lane 2), 8 (lane 3), and 32 (lane 4) hours after initiation of differentiation. Identical bands were detected by BRCA2 mAb 2C9 (left panel) and by anti-BRCA2 rabbit polyclonal serum #5815 (right panel) by Western blot analysis. The reactivity of both antibodies increased transiently, reaching peak levels at 4 hours, and decreasing back to baseline by 32 hours. No bands at this molecular weight were detected by either negative control mAb P3x63 or normal rabbit serum (not shown).

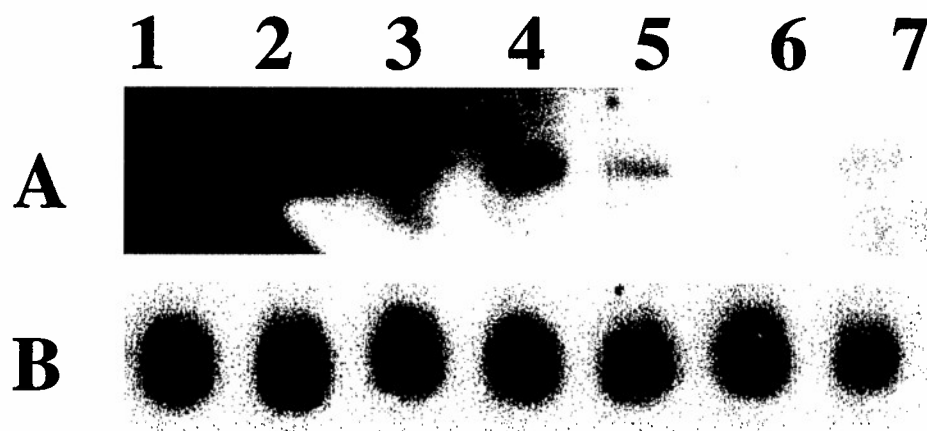
suggesting that this lower molecular weight band is, in fact, BRCA2. These results are not inconsistent with results reported for BRCA1, in which several molecular species are observed on Western blot. Reasons for the discrepancy in size in both cases may include alternative splicing events, differential RNA processing, BRCA2 protein processing, protein degradation, or cross-reactivity. These reasons are discussed more fully later in this chapter.

### **Levels of BRCA2 mRNA Do Not Change Significantly with Differentiation of Keratinocytes.**

Because the immunohistochemical reactivity of 2C9 correlates with terminal differentiation of epithelial cells, we examined BRCA2 mRNA levels in terminally differentiating keratinocytes by Northern blot analysis. BRCA2 mRNA levels do not appear to change during differentiation of keratinocytes, suggesting that the upregulation of protein in these cells is occurring at a post-transcriptional level (Figure 3.16).

### **BRCA2 mAb 2C9 Does Not Cross React With Cytokeratins.**

In addition to comparing reactivity patterns of other anti-BRCA2 antibodies generated against different portions of the molecule to confirm the specificity of 2C9 mAb reactivity for BRCA2, we performed a computer-based analysis of the nucleotide and predicted amino acid sequence for BRCA2, searching for similarities to any known sequences in the databank. Although BLAST searching revealed no significant homology to other differentiation-associated proteins at the nucleic acid level, the 65 aa portion of BRCA2 to which the 2C9 epitope has been narrowed demonstrated limited amino acid homology with the differentiation-associated cytokeratin 2 (CK2) molecule (45% identity, with 76% similarity; longest stretch of identical aa = 3) over a 22 amino



**Figure 3.16: Increasing 2C9 immunoreactivity does not correlate with changes in BRCA2 mRNA levels in differentiating keratinocytes.** We examined BRCA2 mRNA levels by Northern blot analysis in keratinocytes stimulated to differentiate by culture in "complete medium" containing FCS. Differentiating keratinocytes were harvested at 0, 4, 12, 24, 48, 72, or 96 hours after initiation of differentiation (lanes 1-7, respectively). The blot was reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to determine mRNA sample loading. BRCA2 mRNA levels did not change measurably over the timecourse, after correction for loading discrepancies. Similar results were seen in two separate experiments using keratinocytes stimulated to differentiate by culture in high calcium (1.2 mM) medium.

acid stretch (aa #1305-1327) (Figure 3.17A). This degree of similarity with  $\leq 3$  amino acids in a row is less likely to result in cross-reactivity since epitopes are 6-8 amino acids and are usually linear when derived using immunogens produced in bacteria. Nevertheless, because we saw 2C9 immunoreactivity in highly keratinized tissues and since high molecular weight cytokeratins are induced upon differentiation in these epithelial cells, we chose to rule out 2C9 cross-reactivity with cytokeratins in these tissues. Western blot analysis of extracts from cultured epidermal keratinocytes and intact human epidermis with anti-CK mAbs AE1/AE3 and AE2 demonstrates that MAb 2C9 is totally non-reactive with keratinocytes and epidermal extracts under conditions in which mAbs AE1/AE3 and AE2 strongly react with their specific cytokeratins (Figure 3.17B and not shown). In addition, immunohistochemical analysis demonstrates a different staining pattern for mAb 2C9, compared with that of mAbs recognizing CK2, in the epidermis and underlying dermal structures (see figure 3.7). These data suggest that 2C9 is not cross-reactive with cytokeratins in Western blot and immunohistochemical assays.

**Figure 3.17: BRCA2 mAb 2C9 does not cross react with cytokeratins.** Panel A shows BLAST search results demonstrating limited amino acid homology present between the BRCA2 protein (2C9 epitope) and the differentiation-associated cytokeratin 2 (CK2) molecule. The two sequences are 45% identical, and common amino acids are shown in bold type. Of note, the two sequences have no more than 3 sequential amino acids in common. To rule out cross reactivity with cytokeratins, we performed Western blot analysis on epidermal extracts derived as described in Figure 3.10. The protein extracts were analyzed on a 4-20% gradient gel, transferred to nitrocellulose, and reacted with anti-BRCA2 mAb 2C9 (lane 3), anti-cytokeratin mAb AE2 (lane 2), and negative control mAb P3x63 (lane 1) in a Western blot assay. MAb AE2 was strongly reactive with cytokeratins from this tissue at the expected molecular weights of 56.5 and 65.5 kDa (Moll, 1982; Collin, 1992). Cytokeratins comprise a large fraction of the proteins present in epidermis, and the urea extraction method optimized the extraction of these proteins. It was clear from this experiment that mAb 2C9 was non-reactive with the same bands detected by mAb AE2. In fact, negative staining (decreased background) at the molecular weights at which mAb AE2 reacted strongly was observed in both the P3 and 2C9 lanes. A lower molecular weight band of approximately 50 kDa was seen in both the 2C9 and negative control P3 lanes, suggesting that this band is non-specific.

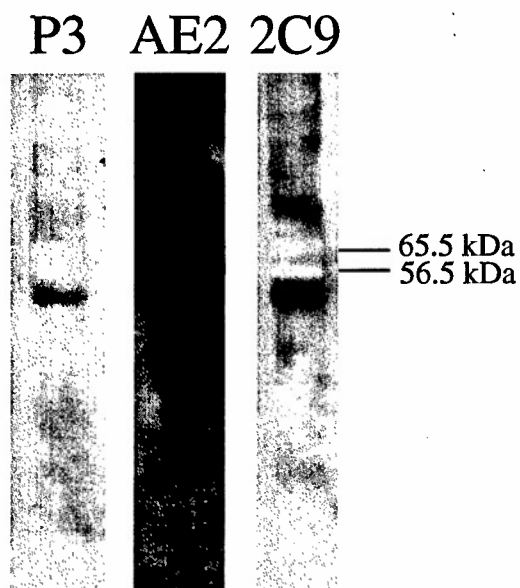
## A) BLAST search results

**BRCA2-aa#1305-1327**

Val-Glu-Glu-Ile-Thr-Glu-Asn-Tyr-Lys-Arg-Asn-Thr-Glu-Asn-Glu-Asp-Asn-Lys-Tyr-Thr-Ala-Ala  
Met-Gln-Asp-Leu-Val-Glu-Asp-Tyr-Lys-Lys-Lys-Tyr-Glu-Asp-Glu-Ile-Asn-Lys-Arg-Thr-Ala-Ala

**CK2-aa#263-284**

**B)**



## Discussion

This study demonstrates that BRCA2 mAbs react with epithelial cells undergoing terminal differentiation, including skin, cultured epidermal keratinocytes, Hassall's bodies in the thymus, and squamous carcinomas. We did not see reactivity with either normal or malignant breast epithelium, suggesting that BRCA2 protein is expressed in these tissues at levels which are undetectable in immunohistochemical analyses. The role of BRCA2 in nuclear activities, primarily DNA damage repair, has been under intensive investigation; however, the involvement of BRCA2 in differentiation is poorly understood. A significant amount of evidence supports the concept that BRCA2 is important in the process of differentiation, in addition to its role in mitogenesis. Initial *in vitro* studies demonstrated that BRCA2 mRNA expression is cell cycle regulated and decreases to negligible levels during serum starvation or at confluency, when cells are no longer proliferating (Rajan, 1996; Vaughn, 1996). Rajan *et al.* further demonstrated that BRCA2 is upregulated in response to lactogenic hormones in post-confluent, *non-proliferative* cells during mammary epithelial cell differentiation (Rajan, 1996). *In situ* hybridization studies in the mouse have demonstrated that BRCA2 is expressed in many tissues in which cellular compartments are undergoing rapid proliferation, including ovary, testis, lymph node, spleen, thymus, pancreas, endometrium, stomach mucosa, intestinal crypts, epithelium in the outer root sheath of hair follicles, and mammary gland, where expression is upregulated during pregnancy, again suggesting that this protein is important in normal control of the cell cycle (Rajan, 1997; Connor, 1997b; Blackshear, 1998). However, Blackshear *et al.* also demonstrated BRCA2 mRNA expression in some non-proliferating cell types, including terminally differentiated neurons and more differentiated cells undergoing spermatogenesis (Blackshear, 1998). These studies



suggest some function for BRCA2 in differentiation processes, unrelated to its involvement in the cell cycle. Further evidence for the role of BRCA2 in the process of differentiation is provided by the phenotype of the mice homozygous for truncated BRCA2 which survive embryogenesis (Connor, 1997a), in which some tissues show evidence of improper differentiation, including skin, testes, ovaries, and thymocytes.

Clearly, BRCA2 has important roles in both cellular proliferation and differentiation; however, the mechanisms involved are still unclear. It is difficult to understand how BRCA2's "proliferative" role, as seen in the non-viable or growth retarded BRCA2-deficient mice, might be related to tumorigenesis in humans. Based on the finding that levels of the cyclin-dependent kinase inhibitor p21<sup>Cip1/WAF1</sup> are increased in BRCA2 mutant embryos, Suzuki *et al.* suggested that the lack of BRCA2 protein in BRCA2-deficient mice results in DNA repair defects during the highly proliferative stages of embryogenesis that then activate pathways involved in checkpoint control, culminating in cell cycle arrest (Suzuki, 1997). Connor *et al.* also found that p21 and p53, are overexpressed in mouse embryonic fibroblasts derived from embryos homozygous for truncated BRCA2 (Connor, 1997a). Furthermore, BRCA2/p53 nullizygotes have a less severe phenotype than mice with BRCA2-deficiency alone (with average survival time *in utero* increased from 8.5 days to 10.5 days), suggesting that the upregulation of p53 noted in the BRCA2-null mice, or some downstream effect of this upregulation, is partly responsible for the earlier embryonic lethality in these animals (Ludwig, 1997).

A model for human tumorigenesis due to loss of BRCA2 has been proposed in which both the loss of the wild-type allele in BRCA2 mutation carriers, as well as loss of the p53 pathway, are required for tumorigenesis (Connor, 1997a; Bertwistle and Ashworth, 1998). This model requires that a mammary epithelial cell in a BRCA2 mutation carrier suffer three "hits" prior to malignant transformation (two hits to knock

out the p53 pathway, and one hit to knock out the wild-type BRCA2 allele.)

Furthermore, it is likely that the p53 pathway would have to be eliminated first, followed by loss of the wild-type BRCA2 allele, as mutations occurring in the opposite order would probably have the same effect as is seen in BRCA2-null mice, namely growth arrest rather than uncontrolled proliferation (Bertwistle and Ashworth, 1998).

Another possibility to consider in trying to understand BRCA2's contribution to tumor suppression is its putative role in the process of differentiation. Most normal cells in an adult are non-proliferative, unless tissue damage or response to particular hormones or mitogens stimulates these cells to re-enter the cell cycle. Furthermore, most cells that are actively proliferating, aside from the pure population of stem cells, are programmed to exit the cell cycle at some point and enter the differentiation pathway. Mammary epithelial cells would be included in the latter category, in that a subpopulation of these cells is hormonally stimulated to develop into differentiated structures in the breast with each menstrual cycle. Thus, unchecked proliferation in these cells may be consequent to either unresponsiveness to signals that control cell cycle activity, or alterations in a signal that would normally instruct these cells to leave the cell cycle and enter the differentiation pathway.

Histochemical analysis of normal and tumor breast tissues from individuals with BRCA2 mutations lends support to the concept that lack of BRCA2 is associated with an alteration in the differentiation capability of these tissues. A recent report found that breast tumors from individuals with the 999del5 BRCA2 mutation were of higher grade than those of an age-matched control group (Agnarsson, 1998). Specifically, these tumors were found to have less tubule formation, more nuclear polymorphism, and increased mitotic frequency, in comparison to control samples. However, in spite of the poorly differentiated appearance of these tumors, these BRCA2-negative tumors were found to have higher levels of steroid hormone receptors (generally associated with well

differentiated tumors). These findings remain to be confirmed, as higher tumor grades in patients with BRCA2 mutations were not observed in another small study (Marcus, 1997).

Expression of BRCA1 has been previously noted to be regulated coordinately with BRCA2, and both BRCA1 and BRCA2 have been shown to interact with the Rad51 protein. These findings have led to the concept that BRCA1 and BRCA2 may function in the same pathway. The observation by Russo and Russo, discussed in Chapter 1, that normal breast tissue from BRCA1 mutation carriers does not undergo normal changes following the first pregnancy (Lob1 and Lob 2 differentiation into Lob 3 structures) (Russo and Russo, 1997), taken together with our data demonstrating a role for BRCA2 in differentiation, lends support to the concept that a lack of BRCA2 may also alter the differentiation pathway in breast epithelial cells. Improper epithelial differentiation in BRCA2 mutation carriers may thus represent a susceptibility factor, similar to that of nulliparity, for the development of breast cancer.

A recent report demonstrated that p21, which has traditionally been considered an inducer of differentiation, is initially upregulated in cultured mouse keratinocytes following a differentiation signal, but that the protein levels must quickly be downregulated in order for differentiation to proceed (Di Cunto, 1998). These data are consistent with a potential role for BRCA2 in a negative feedback mechanism required in differentiating epithelium to eliminate elevated levels of p21 protein and allow differentiation to proceed normally. As discussed previously, normal differentiation does not occur in some tissues from mice homozygous for truncated BRCA2. Of particular relevance to our studies, these mice lack the epidermal stratum corneum, the most differentiated layer of skin (Connor, 1997a). The abnormal differentiation of skin and other tissues noted in these BRCA2<sup>tr/tr</sup> mice may thus be due to the consistent elevation of p21 levels found in these animals. According to this hypothesis, loss of BRCA2

resulting in p21-mediated resistance to normal differentiation processes may be a mechanism for tumorigenesis. Whether p21 is functionally involved in the BRCA2<sup>-/-</sup> mutant phenotype (and by correlation in tumorigenesis in humans); and if so, whether the effect of its upregulated expression is due to an anti-proliferative function or to an inhibition of normal differentiation processes has yet to be elucidated.

Bertwistle *et al.* recently identified BRCA2 protein by immunoprecipitation using multiple polyclonal antisera directed against different portions of the BRCA2 molecule, and detection with a rat monoclonal antibody raised against the C-terminus (aa#3386-3400, exon 27) of BRCA2. A single high molecular weight (MW) species was detected in each case by the rat monoclonal antibody (Bertwistle, 1997). Similarly, Chen *et al.* immunoprecipitated a high MW species that was consistent with full-length BRCA2 from <sup>35</sup>S-labeled T24 bladder carcinoma cells, using each of two BRCA2 polyclonal antisera which were generated using the same immunogens used to generate mAbs 3E6 and 5F6 (Chen, 1998). In cell lines transfected with the full length BRCA2 construct, we detect a single high MW band consistent with the predicted 384 kDa using our monoclonal and polyclonal antibodies. However, we also see lower MW species in addition to the high MW band in immunoprecipitation-Western blot experiments from breast cancer cell lines. Possible mechanisms for the generation of smaller MW species are discussed in the results section. In addition, we see *only* lower MW species in Western blot analysis of human foreskin keratinocytes, with *no* 384 kDa band detected in these cells by straight Western blot analysis (Figure 3.15).

Two possibilities would provide explanations for these findings. In tissues in which BRCA2 is present at a high enough level for detection by the less sensitive immunohistochemical or straight Western blot analyses (as in the case of the foreskin keratinocytes), the lower MW species may be the predominant form of BRCA2 present. Full length BRCA2 may be present, but not detected at this level of sensitivity.

Alternatively, our mAb 2C9 may be particularly reactive with the smaller MW species. If generation of the smaller forms of BRCA2 results in conformational changes in the protein that make the 2C9 epitope more accessible for binding (i.e. via differential post-translational protein processing, protein degradation, or differential splicing), then 2C9 may be more sensitive under conditions in which smaller MW species are present. The fact that exclusively smaller MW species are also detected by the anti-BRCA2 rabbit polyclonal serum in keratinocytes by Western blot (Figure 3.15) suggests that the first explanation, the predominant presence of smaller forms of BRCA2 under certain conditions, is more likely. This may be a differentiation-specific phenomenon. Thus, it is possible that our antibodies detect post-translationally processed BRCA2 species which, if these species did not include the C-terminal portion of BRCA2, would not be detected by the rat monoclonal antibody used in the Bertwistle report.

In both the Bertwistle and Chen reports, these investigators localized BRCA2 protein to the nucleus of carcinoma cells by differential purification of subcellular fractions (Bertwistle, 1997; Chen, 1998). This protein's interaction with RAD51 and its putative role in double stranded DNA repair are consistent with its localization to the nucleus. However, our experiments have consistently demonstrated BRCA2 mAb 2C9 reactivity in the cytoplasm of differentiating cells, in a perinuclear pattern with nuclear dots. Our results may not be incompatible with studies demonstrating nuclear localization of BRCA2 in carcinoma cells, as this molecule may have different functions, and thus different localization, based on the cell type or stage of development. Its function as a molecule involved in the process of differentiation may require its presence in the cytoplasm, whereas its function as a DNA repair molecule, or molecule otherwise involved in cell cycle processes, may require its presence in the nucleus.

As BRCA1 and BRCA2 appear to be coordinately regulated, and mRNA for both genes appears to have a very similar distribution, it is likely that the protein expression

pattern is also similar. A similar ongoing debate over the localization of the BRCA1 protein remains unresolved, with reports of nuclear staining as well as differential trafficking with localization to the cytoplasm in breast cancer cells (reviewed in Bertwistle and Ashworth, 1998). Proponents of the differential cellular trafficking of BRCA1 hypothesis have suggested that different splice variants are targeted to either the cytoplasm or the nucleus, based on whether or not the nuclear localization sequence (which is removed in the BRCA1 $\Delta$ 11b splice variant) is present (Wilson, 1997).

Although BRCA2 mRNA splice variants have not been described at the mRNA level, our data demonstrates that smaller species reactive with BRCA2 mAbs are detected by Western blot under certain conditions, as discussed previously. Even in such cases in which smaller species are detected, we see single bands on Northern blot analysis. This suggests that either alternative RNA splicing is not involved and smaller protein species represent products of post-translational alterations, or that these smaller protein species are products of differentially spliced mRNA variants present at levels not detectable by Northern blot analysis. A putative nuclear localization sequence in exon 27 (codons 3263-3269) has also been reported for BRCA2 (McAllister, 1997), and removal of this domain in differentiating cells may account for cytoplasmic localization, in analogous fashion to that proposed for BRCA1. Thus, absence of the C-terminal portion of the BRCA2 molecule in the differentiating tissues and cells in which we observe high level reactivity of anti-BRCA2 mAb 2C9 may account for both the cytoplasmic localization observed immunohistochemically, as well as the smaller MW species observed on Western blot analysis.

A more recent report suggested that the "nuclear dot" staining pattern of BRCA1 mAbs was actually due to reactivity located in tube-like channels extending into the nucleus, likely originating from the perinuclear endoplasmic reticulum-Golgi complex (Coene, 1997). The authors report that the nuclear tubes are seen in a fraction of the cell

population, that they form a much more extensive branching network in cancer cells compared with non-malignant cells, and that the nuclear invaginations are often seen in close proximity to nucleoli, suggesting a possible involvement in the cell cycle. Coene *et al.*'s description of a perinuclear staining pattern with nuclear dots for BRCA1, with distinct visualization of either nuclear staining or cytoplasmic staining being dependent on the fixation method, is consistent with the immunohistochemical staining pattern we observe with mAbs against BRCA2. However, the subcellular localization of BRCA2 in different cell types deserves further investigation.

In our studies, 2C9 clearly reacts immunohistochemically with a cytoplasmic protein in epidermis, cultured keratinocytes, and thymic epithelium. The possibility that the observed 2C9 reactivity in these cells is entirely non-specific, due to cross reactivity with another protein present in skin, thymus, and differentiating keratinocytes, is very unlikely, as mAb 5F6, which is directed against an entirely separate part of the BRCA2 molecule, reacts with these same cells by immunohistochemistry (Figures 3.8, 3.9, and 3.11). In addition, 2C9, 5F6, and 3E6 react with identical high molecular weight bands on Western blot from skin extracts (Figure 3.10), and 2C9 and polyclonal anti-BRCA2 rabbit serum #5815 react with identical bands from cultured keratinocytes undergoing differentiation (Figure 3.15).

Although BLAST search revealed limited homology of a short 22 amino acid stretch of BRCA2 to cytokeratin 2 (CK2), our data clearly demonstrates lack of 2C9 cross reactivity with cytokeratins in Western blots (Figure 3.17), as well as a different immunohistochemical staining pattern compared with that of mAbs recognizing CK2 (Figure 3.7). Furthermore, CK2 is not found in foreskin epithelium, squamous carcinomas, or their derivative cell lines (Collin, 1992), and cytokeratin staining patterns are exclusively cytoplasmic, with no nuclear reactivity. Thus, while it is impossible to totally exclude immunohistochemical cross-reactivity of 2C9 with CK2 in tissue sections,

amassed evidence suggests that 2C9 is specifically detecting BRCA2 present in the cytoplasm of differentiating epithelial cells.

## Summary

In conclusion, direct study of BRCA2 distribution and function within cells has been limited by the lack of appropriate reagents, as well as the fact that the BRCA2 protein is very large and appears to be expressed at very low levels in most tissues. Utilizing monoclonal antibody 2C9 and polyclonal antibody #5815, developed in our lab, as well as other monoclonal antibodies raised against BRCA2 sequences to confirm specificity, we have demonstrated a potential role for BRCA2 in cellular differentiation of keratinocytes and thymic epithelium. Our data supports research showing that BRCA2 mRNA is upregulated in differentiating cells (Rajan, 1996) and extends these findings to the level of BRCA2 protein expression. Furthermore, our findings are consistent with data which demonstrates that lack of BRCA2 results in abnormalities in differentiating tissues (Connor, 1997a). Whether BRCA2 actually plays a role in the process of differentiation in these tissues, or whether this protein's presence in differentiating tissues is simply a result of accumulation due to extensive crosslinking, enhanced stability, or some other factor, has yet to be elucidated. However, BRCA2 likely plays multiple roles in the diverse processes of cellular proliferation, differentiation, and monitoring of the integrity of DNA.



## **Chapter 4**

# **Passive Specific Immunotherapy for Breast Cancer Targeting the EGFRvIII Tumor Antigen**

## Introduction

The use of tumor-associated antigens as specific targets for directing anti-tumor therapy is a promising concept. In this chapter, I describe research involving one such target antigen, EGFRvIII, which is expressed in a large percentage of breast cancers and other tumor types. First, I studied the expression pattern of this variant growth factor receptor in breast cancer cells as well as other non-tumor tissues. EGFRvIII is expressed predominantly in tumor tissues, with high level expression confined to certain tumor types, such as malignant gliomas. Because of this relative specificity of expression, I proceeded to investigate the possibility of immune-based approaches to breast cancer treatment based on EGFRvIII.

As described in Chapter 1, a variety of different immunotherapeutic approaches are currently under investigation. Active immunotherapy for treatment of cancer has been criticized primarily for the difficulties associated with generation of an effective anti-tumor cytotoxic immune response. In the case of EGFRvIII, this antigen may simply not be immunogenic in breast cancer patients due to a lack of effective antigen presentation for a variety of reasons, as outlined in Chapter 1 as well as later in this chapter. Clearly, generation of a cytotoxic immune response is a complex process, and an alternative means of utilizing the specificity of the immune system while bypassing its restrictions would be extremely useful for cancer therapy.

The strategy I investigated uses the tumor cell-specificity of EGFRvIII as a target and eliminates the strict requirements for the *in vivo* generation of a cytotoxic response against the tumor cell. I have generated a bispecific antibody construct (bsAb) in which a bispecific F(ab')<sub>2</sub> heterodimer against both EGFRvIII and CD3 $\epsilon$ , is genetically engineered using leucine zippers (Kostelny, 1992). Many of the older methods for

developing bsAbs (chemical cross-linking, hybrid-hybridomas, and disulfide exchange) are technically difficult and have produced relatively heterogeneous products (Kostelny, 1992; Van Ravenswaay Claasen, 1993). The method I chose utilizes the fact that heterodimerization of the fos and jun leucine zipper domains is strongly thermodynamically favored over homodimerization. Thus, by linking the jun leucine zipper to the constant region of one antibody and the fos leucine zipper to the other, the probability of forming a bispecific reagent is significantly increased. The result is well-defined, homogeneous, dimeric proteins that are unable to crosslink T cell receptors in the absence of EGFRvIII-bearing tumor cells, thus avoiding complex purification and chemical processes and resulting in decreased potential for toxicity. The development of new generations of bsAbs holds promise for the effective adjunct treatment of those human cancers for which a tumor-specific antigen has been identified (such as EGFRvIII) and a monoclonal antibody has been generated.

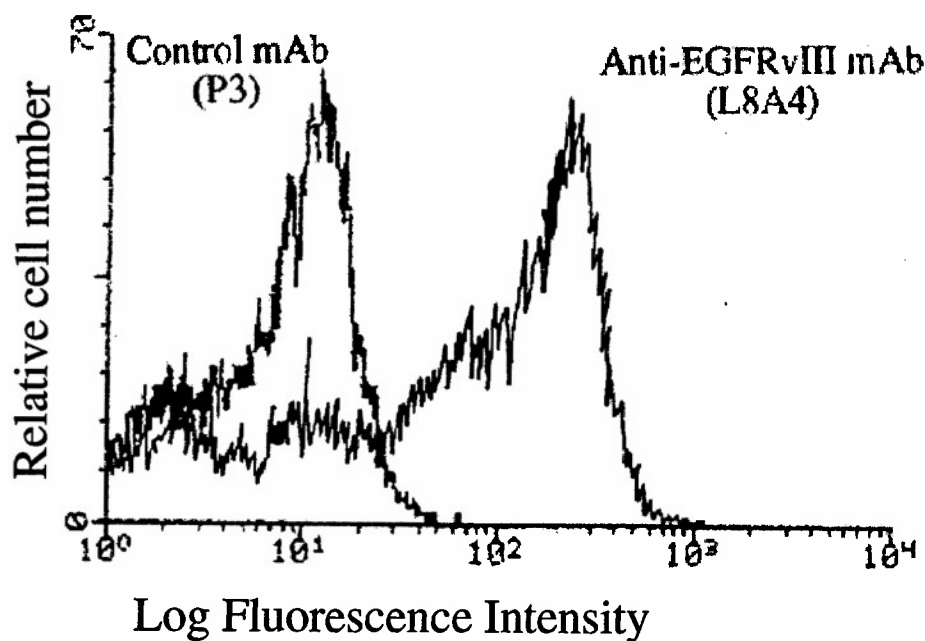
## **Results**

### **Reactivity of EGFRvIII mAb in human tissues**

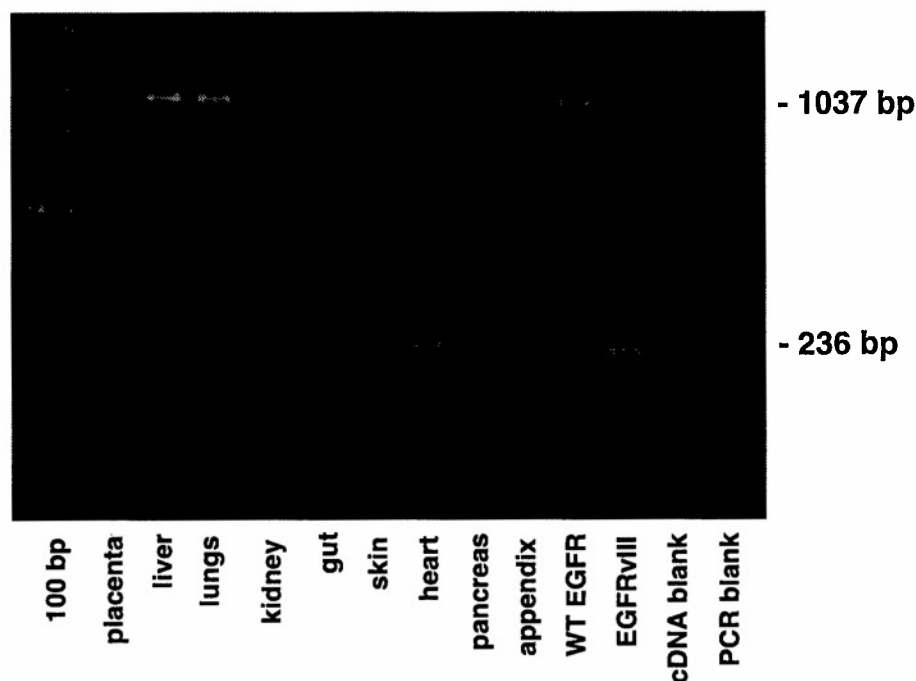
Previous studies examining the tissue distribution of EGFRvIII noted the expression of this protein in a variety of tumor tissues whereas no normal adult tissues were found to express this EGFR variant. Furthermore, RT-PCR analysis generated products specific for the presence of mRNA encoding the EGFRvIII tumor antigen in a high percentage of breast cancers (73%), although only 27% of these tissues expressed protein detected with anti-EGFRvIII mAb by immunohistochemistry, suggesting that most of these tissues express EGFRvIII at low levels (Wikstrand, 1995). To confirm that this reactivity was specific and localized to the surface of the breast tumor cells, we analyzed five fresh, mechanically dissociated breast cancer tissues for reactivity with

anti-EGFRvIII mAb L8A4 by flow cytometric analysis. Tumor cells from all five tissues examined reacted with the anti-EGFRvIII antibody (Figure 4.1). These results confirm that EGFRvIII is a cell surface, tumor-associated antigen which is expressed, albeit at low levels, in a high percentage of breast cancers. Due to the quantitatively lower level of expression of EGFRvIII in these tumors, it is not clear whether EGFRvIII will be a good immunotherapeutic target antigen for breast cancer. However, previous studies have indicated that immunotherapeutic strategies may be effective against even minute amounts of target antigen which are undetectable by flow cytometric analysis (Van Ravenswaay Claasen, 1993). Regardless of whether EGFRvIII will serve as a good target antigen for breast cancer immunotherapy, this antigen will certainly be a good immunotherapeutic target for other tumor types which express higher levels of EGFRvIII. The efficacy of anti-EGFRvIII-expressing tumor targeting is currently being demonstrated in phase I and II passive immunotherapeutic trials in brain tumor patients at this institution (discussed in Chapter 1).

Although EGFRvIII mRNA has not been found in normal adult tissues, products specific for the presence of EGFRvIII mRNA were generated in RT-PCR assays of 16 week fetal tissues, including heart, placenta, liver, and lung (Figure 4.2). These findings suggest that rather than occurring solely as a tumor-specific mutation, EGFRvIII may be an oncofetal antigen normally expressed, perhaps as a splice variant, during fetal development. Very recent flow cytometric data has also suggested that a subset of pediatric human thymocytes may also be reactive with anti-EGFRvIII mAb L8A4. The significance of this reactivity is not clear, as EGFR is not thought to play a role in the development of T-lymphocytes. Thus, these findings require repetition and confirmation by Western blot to rule out possible cross reactivity of mAb L8A4 with another surface antigen. However, even if this observation represents a cross-reactivity in thymocytes rather than reactivity with definitive EGFRvIII, this data suggests that the *reactivity* of the



**Figure 4.1: Detection of EGFRvIII expression in breast cancer tissue by flow cytometric analysis.** Fresh breast cancer tissue was mechanically disaggregated and reacted with EGFRvIII-specific (L8A4) or negative control (P3x63) mAb followed by FITC- goat anti-mouse Ig. The population of breast tumor cells reacted with L8A4 (red histogram) had a mean fluorescence (linear scale) of 47.83, compared with the population stained with the P3 negative control (black histogram), which had a mean fluorescence of 7.17. Data shown are representative of the 5 breast cancer tissues tested.



**Figure 4.2: Detection of EGFRvIII in 16-week fetal tissues by RT-PCR.** mRNA was extracted from fetal tissues and subjected to RT-PCR. Tissues studied included placenta (lane 2), fetal liver (lane 3), fetal lung (lane 4), fetal kidney (lane 5), fetal gut (lane 6), fetal skin (lane 7), fetal heart (lane 8), fetal pancreas (lane 9), and fetal appendix (lane 10). Positive controls included glioma xenograft 727 expressing wild-type EGFR (lane 11), and glioma xenograft D256 expressing EGFRvIII (lane 12). Negative controls included those lacking template (lane 14) or reverse transcription (lane 13). 100 base pair markers are shown in lane 1. Specific products corresponding to EGFRvIII were detected in fetal placenta, liver, and lung, and heart.

antibody L8A4 is not limited to tumor-associated antigens as previously hypothesized (Wikstrand, 1995). It is also possible that this variant form of EGFR is expressed at a low level such that it was not detected in the comprehensive immunohistochemical screen we performed on a panel of normal tissues. The implications for these caveats are summarized in the discussion section of this chapter.

### **Evaluation of an anti-EGFRvIII immune response in breast cancer patients**

The data presented above suggests that a large percentage of breast cancers express EGFRvIII, at least at low levels. We therefore wished to determine whether breast cancer patients have naturally occurring immune responses against EGFRvIII. We assayed a panel of human plasma samples (9 breast cancer patients and 6 controls) for specific antibody responses against Pep-3, a synthetic peptide representing the unique region of EGFRvIII, using an ELISA assay. This study identified one of nine breast cancer patients who had a weakly positive anti-EGFRvIII titer (defined as 2X background reactivity against an irrelevant peptide at a plasma dilution of 1:40) compared with zero of six controls (Table 4.1). These results indicate that, as in the case of many other tumor antigens, a naturally occurring anti-EGFRvIII immune responses is not common. However, the fact that one woman had a weak humoral immune response against EGFRvIII suggests that clonal deletion of all potentially responsive cells is less likely. Alternative explanations for the low percentage of women with breast cancer who generate anti-EGFRvIII immune responses include immuno-evasive mechanisms of the tumor cells or lack of a second signal required for induction of an immune response, as discussed in Chapter 1. In addition, the EGFRvIII tumor antigen may not be expressed at high enough levels in breast carcinoma to induce a naturally-occurring immune response. Further details on why EGFRvIII may not be presented appropriately by the antigen presenting cells of the host immune system are presented below and in Chapter 1.

<u>Sample</u>	<u>a Pep3</u>	<u>a Irr.</u>	<u>Ratio</u>
#1	0.298	0.275	1.08
#2	0.228	0.219	1.04
#3	0.231	0.245	0.94
#4	0.229	0.233	0.98
#5	0.175	0.21	0.83
#6	0.159	0.179	0.89
#7	0.205	0.225	0.91
#8	0.222	0.209	1.06
#9	0.2	0.183	1.09
#10	0.243	0.434	0.56
<b>#11</b>	<b>0.475</b>	<b>0.238</b>	<b>2.00</b>
#12	0.216	0.222	0.97
#13	0.278	0.256	1.08
#14	0.153	0.15	1.02
#15	0.173	0.165	1.05

**Table 4.1: Natural anti-EGFRvIII humoral response in breast cancer patients.** Coded plasma samples from 9 breast cancer patients and 6 controls were examined for anti-EGFRvIII reactivity by ELISA. Plates were coated with 0.25 mg/well of either Pep3 (a 14 amino acid peptide which spans the splice junction of EGFRvIII) or irrelevant peptide AT-2 (which shares no sequence similarity with Pep3). Individual #11, who was identified as a breast cancer patient, demonstrated weak positive reactivity (2.0X background reactivity) against the Pep3 antigen. None of the 6 controls or the other 8 breast cancer patients exhibited positive reactivity against Pep3.



Although it is still possible that proper manipulation of the immune system could result in a very effective active anti-tumor immune response targeting the EGFRvIII tumor antigen (as discussed in Chapter 5), I have investigated an alternative passive immunotherapeutic approach, the generation of a bispecific antibody. This antibody bispecific for EGFRvIII tumor antigen and the CD3 $\epsilon$  T-cell activation antigen takes advantage of the specificity of the cellular immune response, without requiring immunization or the need to actually generate an immune response *in vivo*. This type of therapy would be useful when an active anti-tumor response can not be induced. In particular, therapy utilizing a bispecific antibody holds promise for the period of time after surgery and chemotherapy, when immune responses are blunted, but the tumor load is low and immunotherapy is likely to be most effective.

### **Bispecific Antibody Construction**

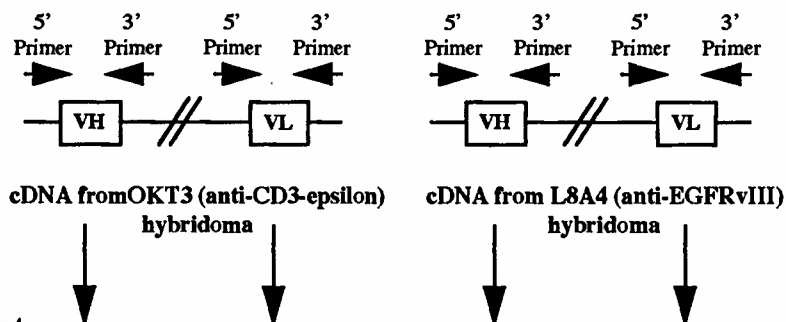
In brief, the strategy used in construction of the bispecific antibody was to link leucine zipper peptides derived from the Fos and Jun proteins to the Fab' portions of EGFRvIII and CD3 $\epsilon$  mAbs by gene fusion using PCR. The original plan was to clone the heavy and light chain variable regions of anti-EGFRvIII mAb L8A4 and anti-CD3 mAb OKT3, construct an Ig heavy chain-leucine zipper fusion gene, and insert these sequences into expression vectors designed specifically for expression of immunoglobulin heavy chain (pSV2 $\Delta$  Hgpt-HuG1) and light chain (pSV184 $\Delta$  Hneo-HuK), obtained from Dr. Sherie Morrison (Batra, 1994).

#### **Strategy One:**

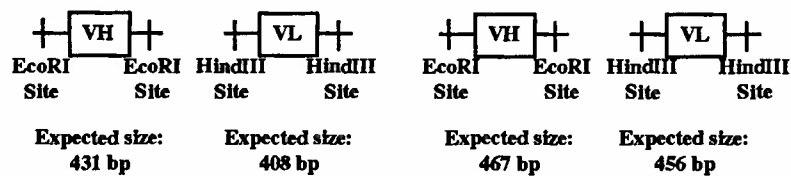
The first step in this strategy was to clone the heavy and light chain variable regions from the anti-CD3 $\epsilon$  hybridoma cell line, OKT3 and from the anti-EGFRvIII hybridoma cell line, L8A4 by RT-PCR (Figure 4.3).

**Figure 4.3: Strategy for PCR amplification and cloning of anti-EGFRvIII and anti-CD3 $\epsilon$  variable regions.** The heavy and light chain variable regions of the monoclonal anti-EGFRvIII (L8A4) and anti-CD3 $\epsilon$  (OKT3) antibodies were cloned as follows: PCR primers were designed based on known variable region sequences, with restriction enzyme sites added to the ends of each primer for future cloning steps. OKT3 heavy chain sequence was obtained from Dr. Robert Zivin. OKT3 light chain sequence was available in Genbank (Accession #A22259). L8A4 heavy and light chain sequences were obtained from Dr. Darell Bigner. RT-PCR analysis was performed using hybridoma RNA, and the PCR reactions were run on a 1.5% agarose gel loaded in the following order: 1) 100 bp markers; 2) OKT3 heavy chain; 3) OKT3 light chain; 4) L8A4 heavy chain; and 5) L8A4 light chain. Separated PCR products were detected by ethidium bromide staining and visualization by UV light. Appropriately-sized products were generated for each of the four primer sets.

## Template:



## Product:



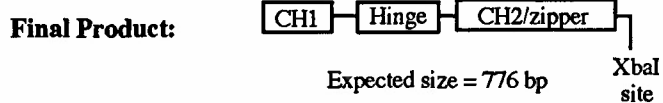
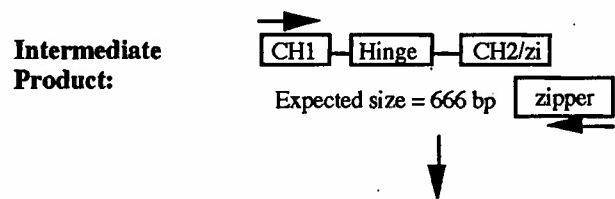
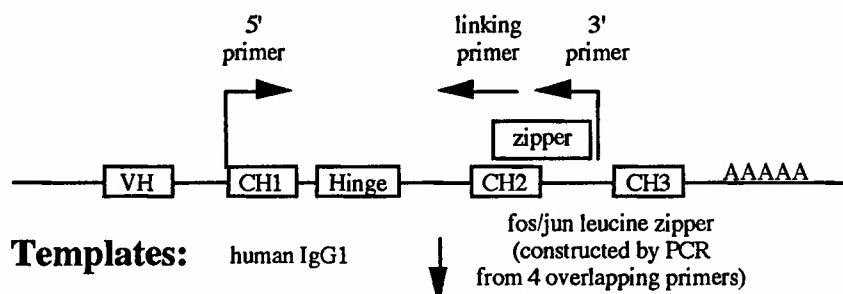
Next, to construct the IgG-leucine zipper fusion gene, digestion at a BstXI site unique in the CH1 region of the human IgG1 sequence was chosen to remove the unnecessary portions of the IgG1 constant region from the intended expression vector and to replace these sequences with the PCR-generated IgG1-leucine zipper fusion gene. Then the specific cloned heavy chain variable region (OKT3 or L8A4) would be cloned into the variable region cloning site to complete the construct. Similarly, the specific cloned light chain variable region would be inserted into the variable region cloning site in the Kappa expression construct.

The fos or jun leucine zipper sequences were successfully joined to the first codon of the C<sub>H</sub>2 exon of the human IgG1 gene (Figure 4.4), using the overlapping PCR method described by Yon and Fried (Yon and Fried, 1989). These IgG1/Fos and IgG1/Jun constructs were then inserted into the PCRII cloning vector. Unfortunately, upon attempted insertion of the completed construct into the pSV2Δ Hgpt-HuG1 expression vector, I found that this vector had several previously unidentified BstXI sites which cleaved preferentially with respect to the desired BstXI cleavage and thus precluded specific insertion of the construct. Furthermore, both the heavy and light chain vectors were very large (>15,000 kb) and were poorly mapped, as their intended use was for the simple exchange of variable region "cassettes" to make chimeric antibodies with specific human isotypes.

#### Strategy Two:

The second strategy for construction of the bsAbs utilized well characterized expression constructs for production of the heavy and light chain proteins *in vitro*. PC1-neo (Promega, Carlsbad, CA) was chosen for the heavy chain, and pcDNA3.1/Hygro (Invitrogen, Madison, WI) for the light chain. Again, the overlapping PCR method was

**Figure 4.4: Construction of a fusion gene (antibody constant region + leucine zipper) by overlapping PCR.** Fusion genes were generated, in which the fos or jun leucine zipper proteins were directly attached to the 3' end of the IgG1 CH2 region by overlapping PCR. The final product of this reaction included sequence encoding the entire CH1 region, the hinge region, the first amino acid of CH2, two glycine residues at the fusion junction for flexibility, and the fos or jun sequences. PCR reactions were run on a 1.5% agarose gel loaded as follows: 1) 100 bp markers; 2) IgG1-fos fusion; 3) IgG1-jun fusion. Separated PCR products were detected by ethidium bromide staining and visualization by UV light. Appropriately-sized products were generated for each reaction.

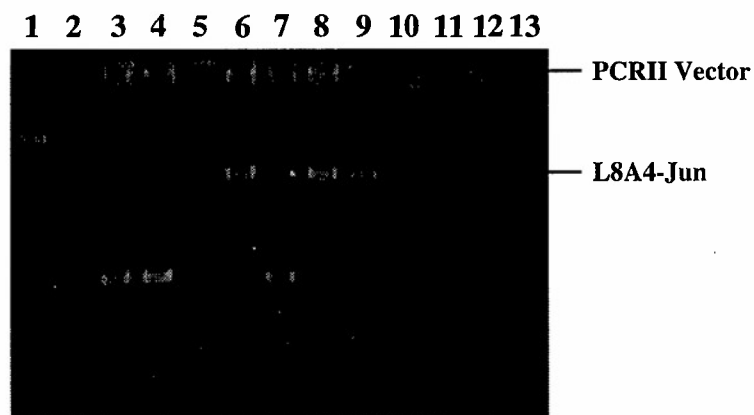
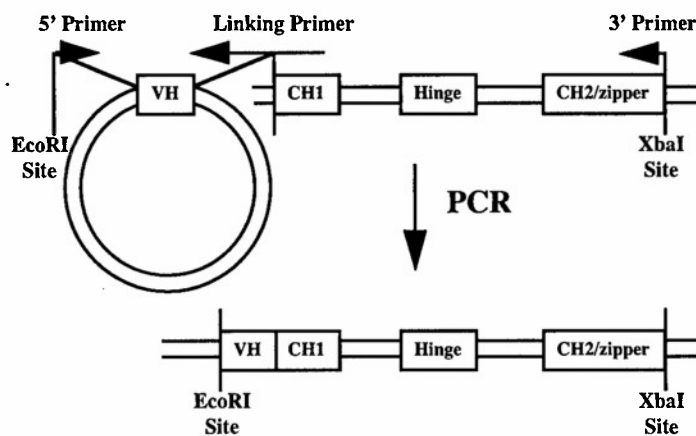


used to join the jun or fos leucine zipper sequence to the first codon of the C<sub>H</sub>2 exon of the human IgG1 gene, this time including the *entire* coding sequence of the C<sub>H</sub>1 and hinge regions and the first codon of the C<sub>H</sub>2 exon. The cloned OKT3 sequence was then directly joined (in a second fusion PCR step) to the beginning of the IgG1-fos fusion gene, with the L8A4 sequence joined to the IgG1-jun fusion gene in an analogous fashion (Figure 4.5a). Similarly, the OKT3 and L8A4 light chain variable regions were directly joined to the human kappa sequence by overlapping PCR (Figure 4.5b).

The fusion junctions of these four constructs were sequenced to rule out PCR-generated mistakes. Both the L8A4-CH1 and the CH2-Jun fusion junctions of the L8A4-Jun construct required corrections. The sequence I had originally used to engineer the L8A4-IgG1 overlapping primer included a 15 amino acid stretch of the mouse CH1 constant region following the end of the actual variable region sequence. To correct this problem, I digested with the restriction enzymes BsmAI and ApaI, which cut the L8A4-Jun sequence uniquely at sites just prior to and following the fusion junction (nucleotides #409 and #476 of the original L8A4-Jun sequence). I then performed a ligation reaction, joining the two fragments with two complementary "adapter" primers encoding the correct fusion junction sequence, as described in Chapter 2. This digestion and ligation thus removed the extraneous 30 amino acids derived from the mouse IgG1 CH1 domain without requiring the repetition of the double fusion PCR reactions using a new overlapping primer. Figure 4.6 presents the strategy used for correction of this junction, the clones derived from the adaptor ligation, and the corrected sequence. The L8A4-Jun construct was also incorrect at the other fusion junction, at the site where the heavy chain C<sub>H</sub>2 region was joined to the jun sequence. This region appeared to be particularly PCR mistake-prone, likely due to a high GC content in that region, as evidenced by the fact that despite several fusion PCR reactions, no clones with the correct sequence were ever generated. Thus, site-directed mutagenesis was performed on one clone which had been

**Figure 4.5a: Joining heavy chain variable region to IgG1/Fos or IgG1/Jun fusion gene by overlapping PCR.** The IgG1-fos or IgG1-jun fusion proteins generated as described in the previous figure, were next joined to the heavy chain variable region of each antibody. The IgG1-jun construct was fused to the L8A4 heavy chain variable region, and the IgG1-fos construct was fused to the OKT3 heavy chain variable region. PCR products were T-A cloned into the PCRII vector, and clones were screened for appropriate-sized inserts. Miniprep DNA for both constructs was restriction enzyme-digested with EcoRI and XbaI to isolate the inserts for a direct size comparison. Digested miniprep DNAs were run on 1.5% agarose gels, loaded as follows: L8A4-jun miniprep DNA clones #2, 3, 4, 5, 6, 7, 9, 11, 12, 13, and 15 were loaded in lanes 2-12 (top gel), and OKT3-fos miniprep DNA clones #1-10 were loaded in lanes 3-12 (bottom gel). PCRII vector, also digested with EcoRI and XbaI, was loaded in lanes 13 (top) and 2 (bottom). 100 bp markers were loaded in lane 1 in both gels and lane 14 (bottom). Lambda DNA/EcoRI+HindIII markers were loaded in lane 13 (bottom). DNA bands were detected by ethidium bromide staining and visualization by UV light. L8A4-jun clones #6, 9, 11, and 13 (lanes 6, 8, 9, and 11) were the correct size (1451 bp), and all of the OKT3-fos clones except #4 (lane 6) were the correct size (1392 bp).



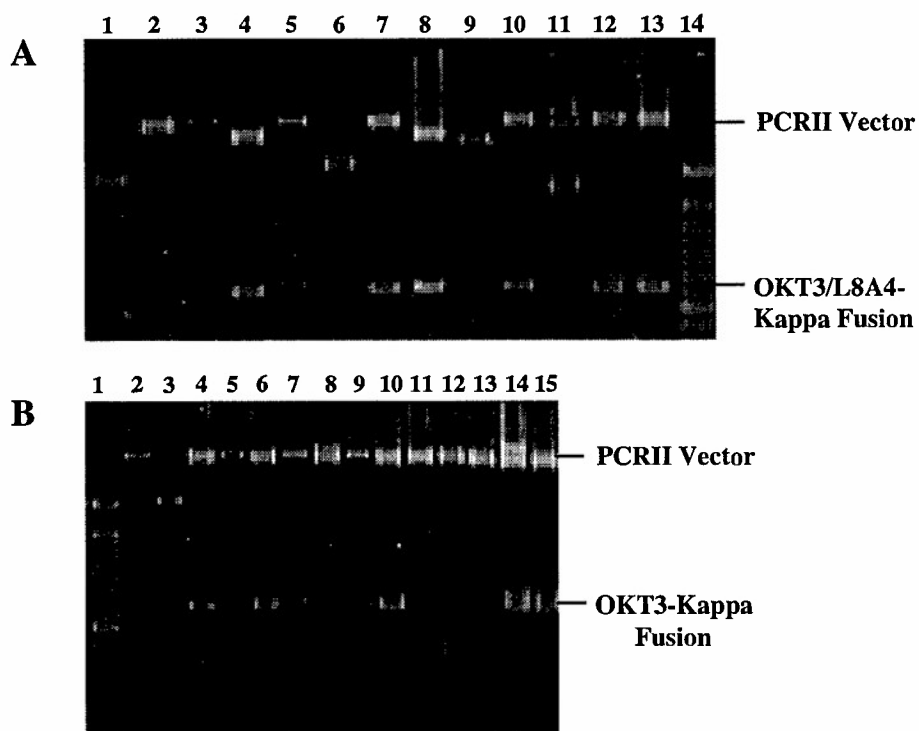
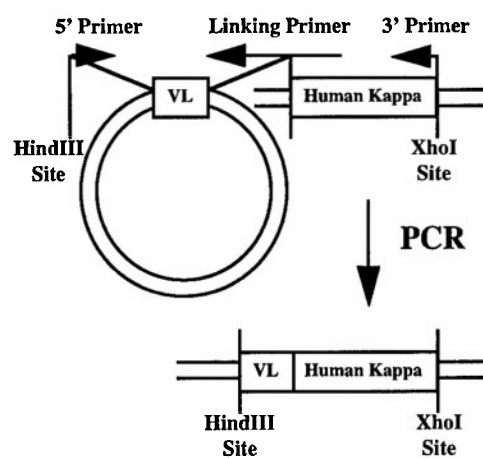


**L8A4-Jun Miniprep DNA**

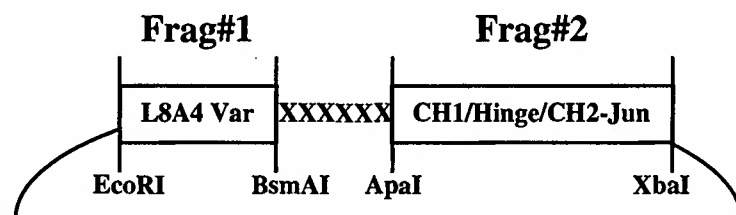


**OKT3-Fos Miniprep DNA**

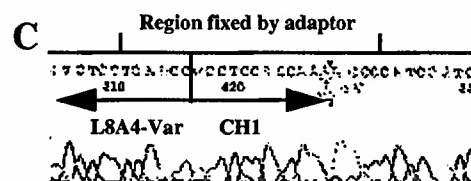
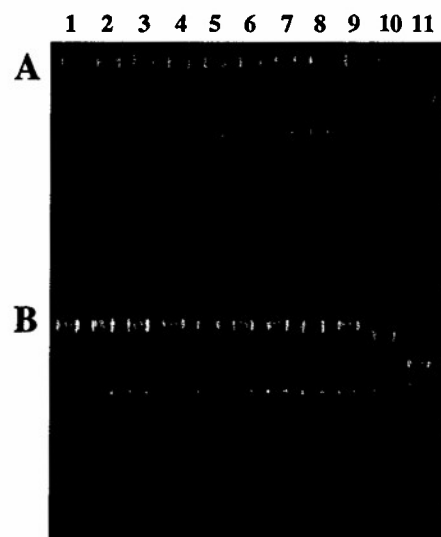
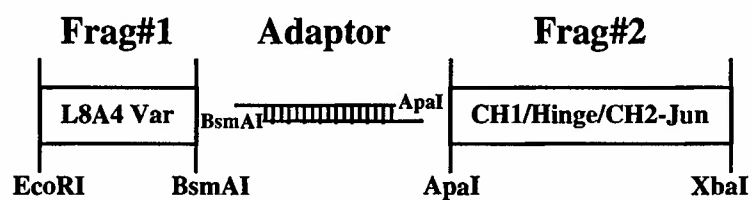
**Figure 4.5b: Joining light chain variable region to human kappa gene by overlapping PCR.** The human kappa sequence was directly fused to the light chain variable region of each antibody in an identical manner to that described in the previous figure (Figure 4.5a). Miniprep DNA for both constructs was restriction enzyme-digested with HindIII and XhoI to isolate the inserts for a direct size comparison. Digested miniprep DNAs were run on 2% agarose gels, loaded as follows: L8A4-Kappa clones #1-10 were loaded in lanes 2-11 (top gel); OKT3-Kappa clones #1-1 and 1-2 were loaded in lanes 10 and 11 (top gel), and clones #1-3 through 1-8 and 3-1 through 3-8 were loaded in lanes 2-15 (bottom gel). 100 bp markers were loaded in lane 1 of both gels and lane 14 (top). DNA bands were detected by ethidium bromide staining and visualization by UV light. L8A4-kappa clones #2, 4, 6, 7, and 9, (lanes 3, 5, 7, 8, and 10) were the correct size (741 bp); and OKT3-kappa clones #1-1, 1-2, 1-3, 1-5, 1-6, 1-7, 1-8, 3-2, 3-3, 3-7, and 3-8 (lanes 12 and 13 - top; lanes 2, 4, 5, 6, 7, 9, 10, 14, and 15 - bottom) were the correct size (732 bp).



**Figure 4.6: Correction of the L8A4 heavy chain variable region-IgG1 fusion junction: Strategy for adaptor ligation, screening of clones, and confirmation of the corrected sequence.** In order to remove the undesired mouse heavy chain sequence from the L8A4-jun constructs (designated by XXXXXX in the diagram), I designed a scheme by which the undesired sequence would be removed from the construct by restriction enzyme digestion with BsmAI and ApaI, which cut on either side of the extraneous sequence. The correct sequence (22 bp long) would then be inserted in the form of an "adaptor," a double-stranded oligonucleotide with "sticky" ends complementary to the BsmAI/ApaI-digested L8A4-Jun construct. These manipulations were performed as described in Chapter 2, and linearized miniprep DNA clones were analyzed by size separation on a 1.5% agarose gel, loaded as follows: L8A4-Jun clones #1-1 through 1-9 and 2-1 through 2-9 were loaded in lanes 3-11 (gels A and B, respectively). Lane 10 of the gel A contained linearized PC1-neo, the vector into which all clones were transferred in the ligation reaction. Lane 10 of gel B contained an "unfixed" clone of L8A4-jun, in the PCRII vector. Lanes 11 of both gels contained 100 bp markers. DNA bands were detected by ethidium bromide staining and visualization by UV light. Size separation was not very informative, as the difference between the old and the new constructs was about 40 bp. However, bands of the appropriate size were obtained for all clones except #1-1, 1-2, and 1-3 (lanes 1-3, gel A). Sequencing confirmed that the overlap region had, in fact, been repaired by adaptor cloning, as shown in panel C.



**L8A4-Jun in PCRII Vector**



corrected at the other fusion junction by adaptor ligation (L8A4-IgG1), to correct the PCR-generated mistake at the C<sub>H</sub>2-Jun junction (Figure 4.7). After confirming that the sequences at all of the fusion junctions were now correct, the assembled constructs from the PCR-II cloning vectors were removed and inserted into the appropriate expression vectors (Figure 4.8).

### **Screening for Production of bsAb proteins**

To screen for clones that produced protein of the appropriate molecular weight for the heavy and light chain constructs, the constructs were transfected individually into LP-293 cells. Unfortunately, clones from only one of the four constructs (OKT3-kappa) produced protein that was detectable by Western blot (Figure 4.9). Furthermore, co-transfection of the L8A4-Jun construct #M7 (the sequence of which was corrected by site-directed mutagenesis) with each of the three L8A4-Kappa constructs (#6A, 7A, and 9A) into SP2/0 myeloma cells produced clones which were resistant to the selection agents encoded by the vectors used, but no immunoglobulin was detectable either in the supernatant or in the cells themselves by Western blot or ELISA. This suggests that the transfection was successful, but that one or both of the constructs was not correct. Of note, the reagent used in the Western blot detection step, HRP-conjugated goat anti-human F(ab)<sup>2</sup>, was not strongly reactive with the F(ab)' portion of the heavy chain (the CH1 region), as was evidenced by the faint signal for heavy chain seen in the positive control lane for normal human serum Ig (Figure 4.9). I concluded that PCR-generated mistakes must exist, at least in the L8A4-Kappa constructs, in regions other than the fusion junctions. Thus, all of the constructs were fully sequenced to pinpoint the source of the problem.

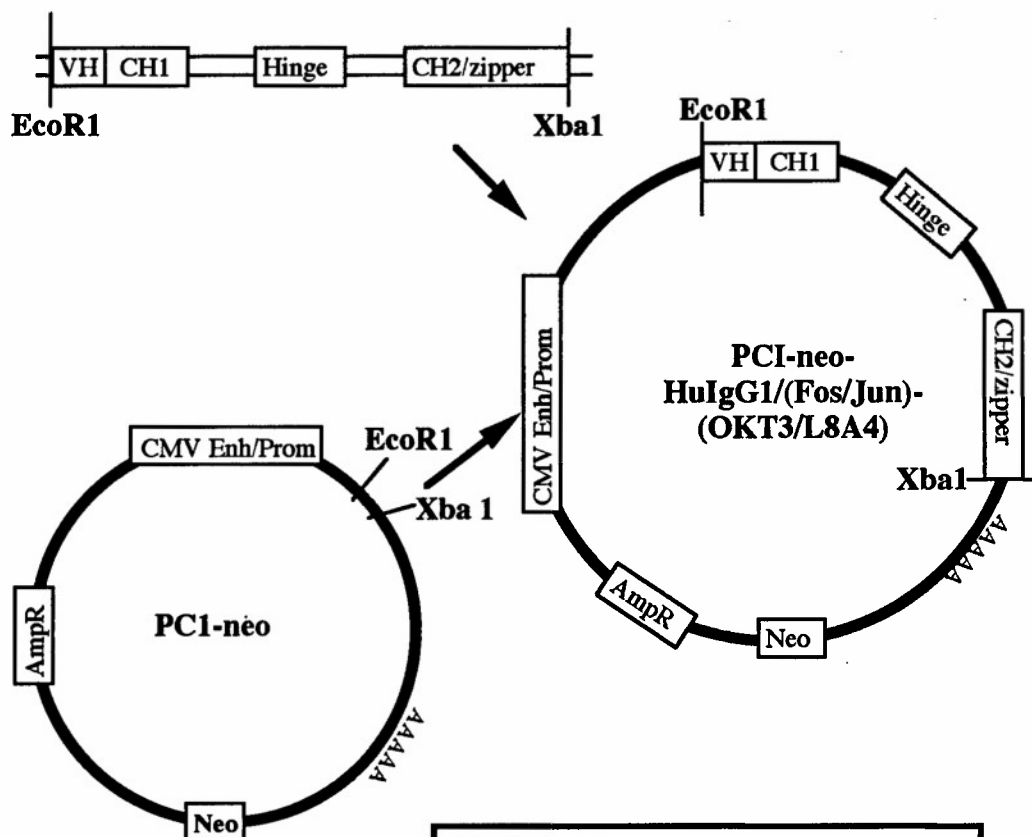
**Figure 4.7: Correction of the IgG1 CH2-Jun fusion junction: Strategy for site directed mutagenesis, screening of clones, and confirmation of the corrected sequence.** Site directed mutagenesis was performed as described in Chapter 2 to correct the PCR-mistake-prone overlap region (denoted by XX) in the L8A4-jun construct. Panel A depicts the strategy used for site directed mutagenesis. Complementary oligonucleotide primers with the correct sequence for this problem area were used to generate copies of the entire vector, using the high fidelity, thermostable enzyme Pfu DNA polymerase. The original bacterially-derived sequences were then fragmented by digestion with DpnI, which cuts bacterially-derived methylated DNA, but not PCR-generated DNA. The reaction mixture, including newly generated, corrected L8A4-jun constructs, were then transformed into bacteria, followed by screening for corrected clones. Digestion with EcoRI and NotI allowed differentiation between uncorrected and corrected clones, as a NotI site present at the site of the incorrect sequence was eliminated when the constructs had been corrected. Analysis by agarose gel electrophoresis (panel B) indicated that all of the clones tested except one (lane 3) were corrected for the mutation, as was evidenced by the 100 bp band seen in this lane, demonstrating that the NotI site was still present. Lane 1 contained the uncorrected L8A4-jun construct, and lanes 2-11 contained clones #1-10 from the mutagenesis. 500 bp markers were loaded in lane 12. Panel C shows sequence for one clone (#M7) confirming that the CH2-Jun overlap region was now correct.





**Figure 4.8: Construction of heavy and light chain immunoglobulin expression vectors.** A) Heavy chain immunoglobulin expression vectors were generated by ligation of an EcoRI/XbaI fragment containing either the L8A4-Jun or the OKT3-fos fusion construct into the PC1-neo vector. B) Light chain immunoglobulin expression vectors were generated by ligation of an HindIII/XhoI fragment containing either the L8A4-Kappa or the OKT3-Kappa fusion construct into the pcDNA3.1/Hyg vector.

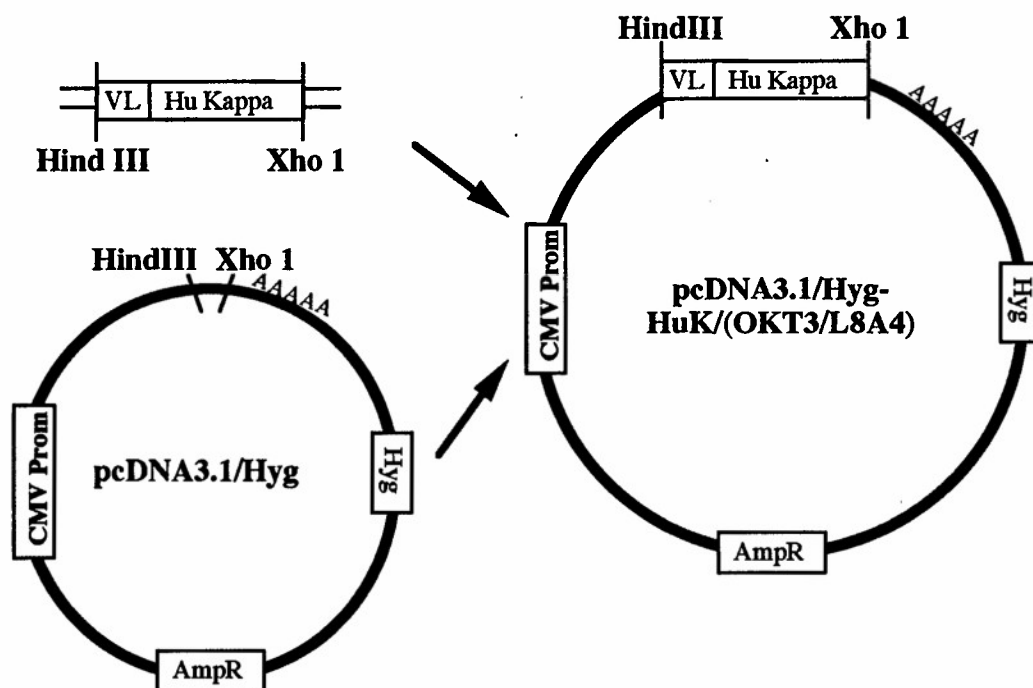
# A



## Legend:

AmpR = Ampicillin resistance gene  
 Neo = Neomycin resistance gene  
 Enh/Prom = CMV-derived enhancer and promoter  
 VH = Heavy chain variable region  
 CH1, Hinge, CH2 = Beginning of Ig constant region  
 Zipper = Fos or Jun leucine zipper sequence

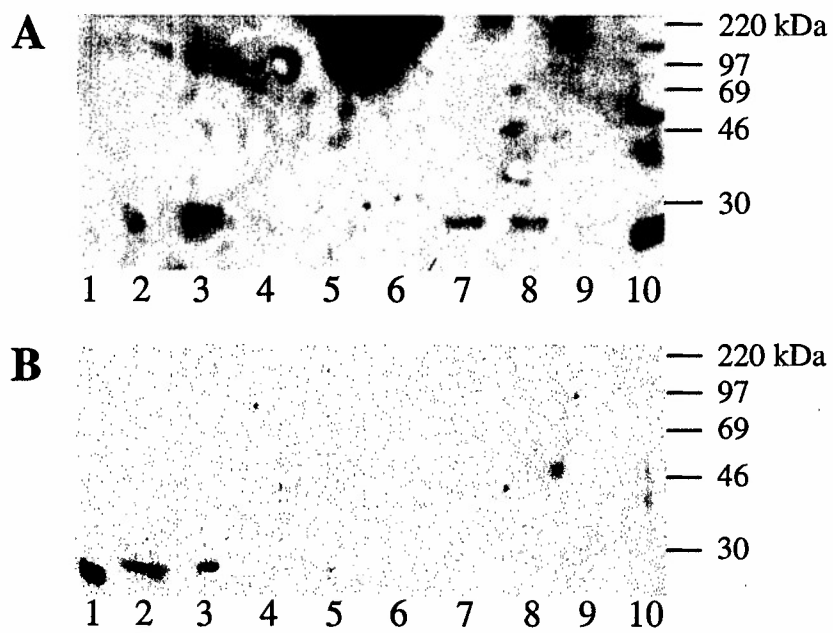
**B**



**Legend:**

AmpR = Ampicillin resistance gene  
 Hyg = Hygromycin resistance gene  
 Prom = CMV-derived promoter  
 VL = Light chain variable region  
 Hu Kappa = Human light chain constant region (kappa)

**Figure 4.9: Screening L8A4-Jun, L8A4-Kappa, OKT3-Fos, and OKT3-Kappa clones for protein-producing constructs.** Lysates from LP-293 cells transfected with various bispecific antibody clones were reacted with goat anti-human-F(ab)' Ig in Western blot assays. Lanes were loaded as follows: OKT3-Kappa #1-1A, 1-2A, 1-3A, 1-5A, 1-6A, 1-7A, 1-8A, 3-2B, 3-3A (lanes 1-9, panel A); OKT3-Kappa #3-7A, 3-8A (lanes 2 and 3, panel B); OKT3-Fos #1B, 2A, 7B (lanes 4-6, panel B); L8A4-Kappa #6B, 7B, 9B (lanes 7-9, panel B); L8A4-Jun #M7 (lane 10, panel B). Markers plus positive control human Ig (reduced with dithiothreitol to separate heavy and light immunoglobulin chains) were loaded in lanes 10 (panel A) and 1 (panel B). Bands of the appropriate molecular weight for Ig light chain, 25 kDa, were seen in lanes containing OKT3-Kappa clones #1-2A, 1-3A, 1-8A, 3-2B, 3-7A, 3-8A (lanes 2, 3, 7, and 8 in panel A; lanes 2 and 3 in panel B) as well as in lanes containing human Ig control (lane 10, panel A; lane 1, panel B). No bands were seen in any lanes containing the L8A4-Kappa, L8A4-Jun, or OKT3-Kappa constructs. Clone **OKT3-Kappa #1-8A** (lane 7, panel A) was selected for DNA maxiprep for use in future experiments.

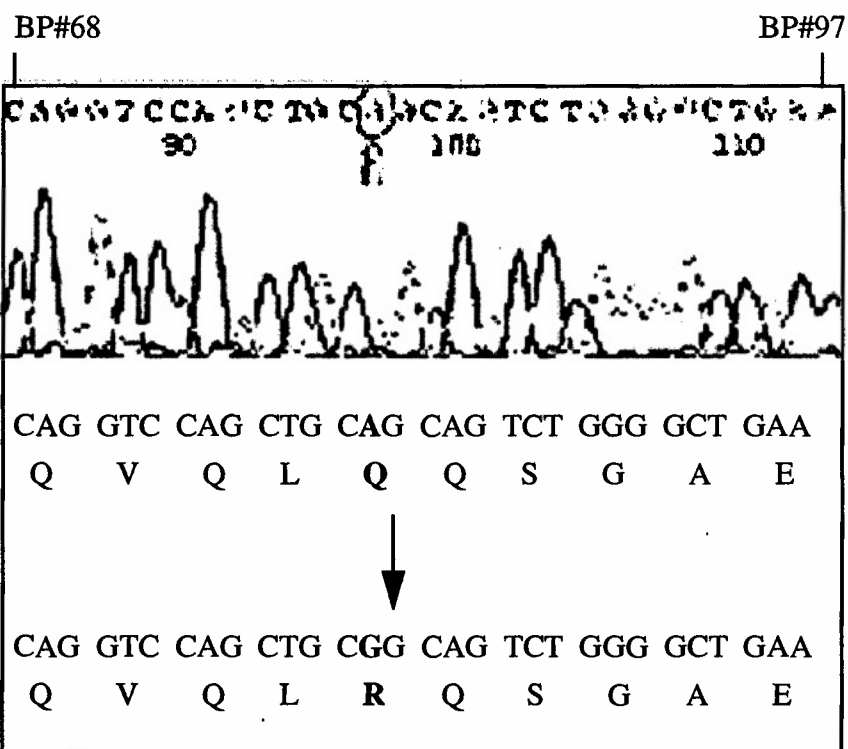


### **Confirmation and Correction of Construct DNA Sequences**

The sequence for OKT3-Kappa#8, which expressed protein detectable by Western blot, was correct, as was the sequence for L8A4-Jun #M7. The OKT3-Fos sequence was found to be inserted backwards into the PC1-neo vector. This was easily corrected by digesting with EcoRI and XbaI, the enzyme sites flanking the fusion sequence, and re-inserting this fragment into EcoRI/XbaI digested PC1-neo, followed by screening for the correct orientation (Figure 4.10). However, sequencing additionally revealed one PCR-generated mistake in the OKT3-Fos construct at base pair #81, which would result in a glutamine to arginine change at amino acid #24 in the variable region. The implications of this amino acid change are unclear at this point. If the CD3 $\epsilon$  binding ability of this antibody is affected by the mutation, site directed mutagenesis will be performed on the construct to correct the mistake.

The L8A4-Kappa light chain sequence had multiple PCR-generated mistakes, precluding straightforward site-directed mutagenesis-based correction. However, an expression vector containing the L8A4 light chain variable region and human kappa sequence has recently been constructed in the laboratory of our collaborator, Dr. Darell Bigner, for other purposes. This expression vector will therefore be used in conjunction with the three constructs generated as described above for production of an anti-EGFRvIII/anti-CD3-epsilon bispecific antibody. Thus, despite the unanticipated difficulties which arose in preparation of these bsAb molecular constructs, the constructs have been completed and will potentially provide a very effective means of passive immunotherapy for patients with EGFRvIII-bearing tumors.

**Figure 4.10: OKT3-Fos fusion construct: identification of PCR-generated mistake in variable region sequence.** Upon sequencing the entire coding regions for the bispecific antibody constructs, a point mutation was found in the OKT3-fos sequence at bp#81. This change would result in a Q-R amino acid change. It is not clear whether this mutation will affect the binding ability of the antibody.

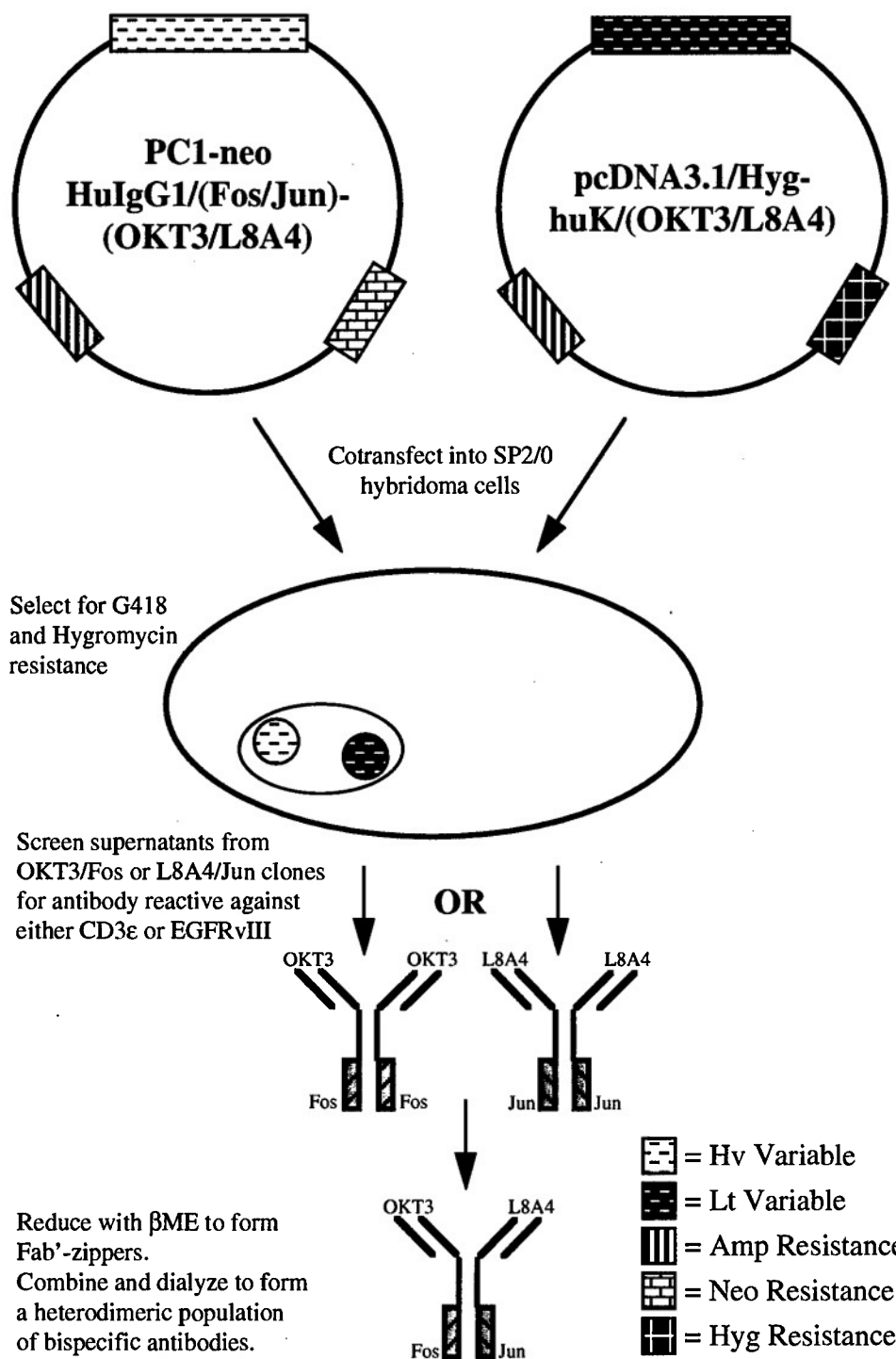




## **Final Strategy**

After these constructs have been produced and transfected into myeloma cells, culture supernatant will be screened for anti-EGFRvIII or anti-CD3 $\epsilon$  activity followed by purification, heterodimerization, and characterization of the bsAb products (Figure 4.11) (Kostelny, 1992). Once the bsAbs have been generated, they will be tested using cytotoxic T-lymphocyte (CTL) assays in which non-specific effector cells are incubated with EGFRvIII-expressing target cells. Lysis of the target cells will suggest that we have, in fact, bypassed the specificity of the T-cells in generating this cytotoxic response. The result of these genetic manipulations will be a product that may potentially be administered to patients with breast cancer, or other EGFRvIII-expressing tumor types (including brain tumors where this antigen is often highly expressed), particularly as adjuvant therapy to effect immune-mediated destruction of metastases.

**Figure 4.11: Strategy for transfection of immunoglobulin expression vectors into SP2/0 hybridoma cells and production of an antibody bispecific for EGFRvIII and CD3-epsilon.**



## Discussion

As explained in Chapter 1, an immunotherapeutic approach which induces an active response is preferable to but more challenging than passive immunotherapy. In the case of the EGFRvIII tumor antigen, successful active immunotherapy requires that T cells with TCRs specific for EGFRvIII exist, that the host APCs are capable of appropriately presenting EGFRvIII-specific epitopes, and that presentation of this antigen occurs in a context in which required second signals are provided to activate the cellular arm of the immune system. The data presented in this chapter suggest that EGFRvIII is expressed in fetal tissues and possibly in pediatric thymocytes, bringing to question whether EGFRvIII will be an appropriate target for active immunotherapy. If EGFRvIII is present as an oncofetal antigen in the thymus when the immune repertoire is being established, reactive-T cell precursor clones would be eliminated, creating a "hole in the repertoire" with respect to EGFRvIII. Consequently, it would be impossible to generate active immunity against this antigen.

Another barrier to the generation of active immunity against this tumor antigen is the limited number of epitopes available for an anti-EGFRvIII response and the possibility that none of an individual's MHC molecules can bind to any of these epitopes. EGFRvIII is identical to wild-type EGFR, with the exception of the novel sequence created at the fusion junction. The range of anti-EGFRvIII-specific epitopes is therefore limited to the number of possible combinations of amino acids spanning this junction. In addition, the EGFRvIII-specific epitopes against which an individual can respond is further limited by the specific binding requirements of their class I MHC molecules. Thus, even if potentially responsive T-cells exist in an individual's repertoire, they may not have class I molecules capable of binding to any of the available EGFRvIII-specific

peptides. Consequently, they would not generate an active anti-tumor immune response if stimulated with their own antigen presenting cells. We have evidence suggesting that one of these mechanisms (a hole in the T-cell repertoire or ineffective antigen presentation) is operative in some mouse strains, in which T-cell responses are not generated against the EGFRvIII epitope under conditions in which an immune response *is* generated against other epitopes present in both the EGFR and EGFRvIII molecules (Ashley, 1997). The current data does not allow us to distinguish between these possibilities. However, this would be an important distinction to make if considering active immunotherapy against EGFRvIII in humans. If EGFRvIII is expressed at such a time and manner that all potentially reactive T cells have been deleted during negative selection in the thymus, then active immunotherapy is not an option. However, if reactivity or non-reactivity against EGFRvIII is determined by the MHC haplotype of an individual patient, then at least a subset of patients would potentially respond to an active immunotherapeutic protocol.

Assuming that an individual is capable of generating an active immune response against EGFRvIII, as is suggested by the naturally-occurring antibody response seen in one breast cancer patient, the concern with using a target antigen which is not confined exclusively to tumor cells is the loss of specificity of the therapy. In passive immunotherapeutic protocols, the loss of specificity translates into therapeutic toxicity, as is seen for other relatively non-specific forms of therapy, such as chemotherapy and radiation therapy. This therapeutic toxicity is likely to be minimal, however, if the target antigen is predominantly found on tumor cells. For active immunotherapeutic protocols, the major concern with targeting a non-specific tumor antigen is the possible generation of an auto-immune response against normal cells bearing the target antigen. This concern has been debated in the literature, and the consensus seems to be that although a tumor-specific antigen is preferable for use as a target in immunotherapeutic approaches, this is

not an absolute requirement. The most important criteria for a target antigen is that it be expressed at much higher levels in the malignant cells than anywhere else in the body. Such tumor non-specific antigens have been used successfully in immunotherapeutic protocols, including those generating active immunity (Anichini, 1993).

In light of the complexities involved in generating an active specific immune response, bispecific antibodies provide an excellent alternative approach for immune-based therapy. The mechanism by which bispecific antibodies function in an anti-tumor response combines the efficacy of the cellular arm of the immune system and the specificity of its targeting action (destruction only of target cells against which the effector cell has been activated), with the advantage of a passive approach which does not require active generation of an immune response. In addition, a bsAb approach to anti-tumor therapy may not be as "passive" as originally believed. Hombach *et al.* have shown that treatment with bispecific antibodies is associated with a bystander effect, in which site-specific cytotoxic activity is induced by autocrine and paracrine T<sub>H</sub>1 cytokine secretion (Hombach, 1997). This effect was shown to result in the recruitment of MHC-independent lymphokine-activated killer (LAK) cytotoxicity, regardless of whether the bispecific antibody was present. Consequently, not only does the bsAb directly cause MHC-independent cytotoxicity, but it also recruits an MHC-independent host response. Thus, in addition to the use of bsAbs as therapeutic reagents specific for antigen-expressing tumor cells, the actual *in vivo* effects of this therapy may also result in a LAK-like response generated against antigen-negative bystander cells. In addition, activation of CD4<sup>+</sup> cells (the cells responsible for the T<sub>H</sub>1 cytokine secretion) via their CD3 complex could potentially result in a long-lasting response. These aspects of bsAb function deserve further investigation.

One disadvantage to using murine-derived antibodies as therapeutic agents in humans is the possibility that cross-species hypersensitivity reactions may occur, due to

the production of human anti-mouse antibodies (HAMAs). This problem has been encountered with other types of therapy which utilize mouse monoclonal antibodies, and the associated problems may not only limit the number of administrations of the agent which may be given but may also pose life-threatening danger if a pre-sensitized person is re-treated. The solution to this problem has been to genetically alter the monoclonal antibodies which are used for human therapies by either chimerization or humanization of the antibodies. Chimerization utilizes the mouse-derived variable regions and combines these with human-derived constant regions, against which patients will not develop an anti-Ig immune response. Humanization takes this one step further, using site-directed mutagenesis to eliminate mouse-specific residues in the variable portion of the mAb gene, including the framework (non complementarity-determining) portions of the variable region, and replacing these with the corresponding human sequence. Although this further decreases the chance of generating a HAMA reaction, these manipulations also often decrease the binding affinity of the monoclonal antibody for its target antigen. In addition, this approach is time consuming and expensive. For this reason, my approach utilized chimeric antibodies, with non-manipulated mouse variable regions sequences and human constant region sequences (kappa, and the first few domains of the IgG1 constant region, including CH1, hinge, and the first amino acid of CH2), as well as human Fos and Jun leucine zipper sequences.

In addition to HAMA-associated toxicities, several studies have demonstrated toxicity associated with the generalized administration of T-cell activating immunological reagents, such as mAb OKT3 and derivatives of this monoclonal antibody (including many bispecific antibodies). This toxicity is attributed to generalized T cell activation secondary to crosslinking of CD3 on host T-cells with Fc receptors on host monocytes and macrophages, which bind the Fc region of the therapeutic antibody reagent. Thus, elimination of the Fc portion of the antibody construct significantly reduces the toxicity

associated with generalized administration of these reagents (Mack, 1997). Newer reagents, including engineered single-chain antibodies, eliminate this problem. Similarly, the construct I have generated eliminates a large part of the constant region of these antibodies, including the Fc portion. Thus, toxicity associated with Fc-TCR conjugation *in vivo* should not be a problem in this case.

Another problem encountered with bispecific antibodies has been the need for activation of the T-cells involved in the anti-tumor response in order to induce effective cytolytic killing. In some cases, conjugation of the TCR with the tumor antigen is not sufficient to generate a killing signal, although there appears to be variability in the efficacy of different anti-CD3 antibodies (Jacobs, 1997). This problem has commonly been addressed by *ex vivo* preactivation of autologous T cells with T cell stimulatory agents, such as IL-2 or OKT3. More recently, costimulation with a second bispecific antibody directed against CD28 and the tumor antigen has been used successfully in animal models, providing the second signal necessary for effective activation of a cytotoxic response (Katayose, 1996; Mazzoni, 1996). Thus, it will likely be necessary either to pre-activate T cells *ex vivo*, or to generate an anti-EGFRvIII/CD28 reagent for co-administration to achieve effective *in vivo* use of the anti-EGFRvIII/anti-CD3 $\epsilon$  bispecific antibody.

## Summary

These studies demonstrate that EGFRvIII is expressed in a subset of breast cancers and may potentially be a suitable target for immunotherapy. I have engineered constructs encoding immunoglobulin heavy and light chains specific for EGFRvIII and CD3 $\epsilon$ , with replacement of the tail end of the heavy chain constant regions with the fos or jun leucine zipper sequences. These reagents can be used to generate bsAbs which



redirect non-specific T cells against EGFRvIII-bearing tumor cells. Production and characterization of these bsAb reagents may allow the development of specific, non-toxic, and effective therapeutic and/or preventative immune-based approaches to breast cancer treatment. Furthermore, this approach may be generalized to other tumor antigens for broad applicability to a large variety of tumor types.

## **Chapter 5**

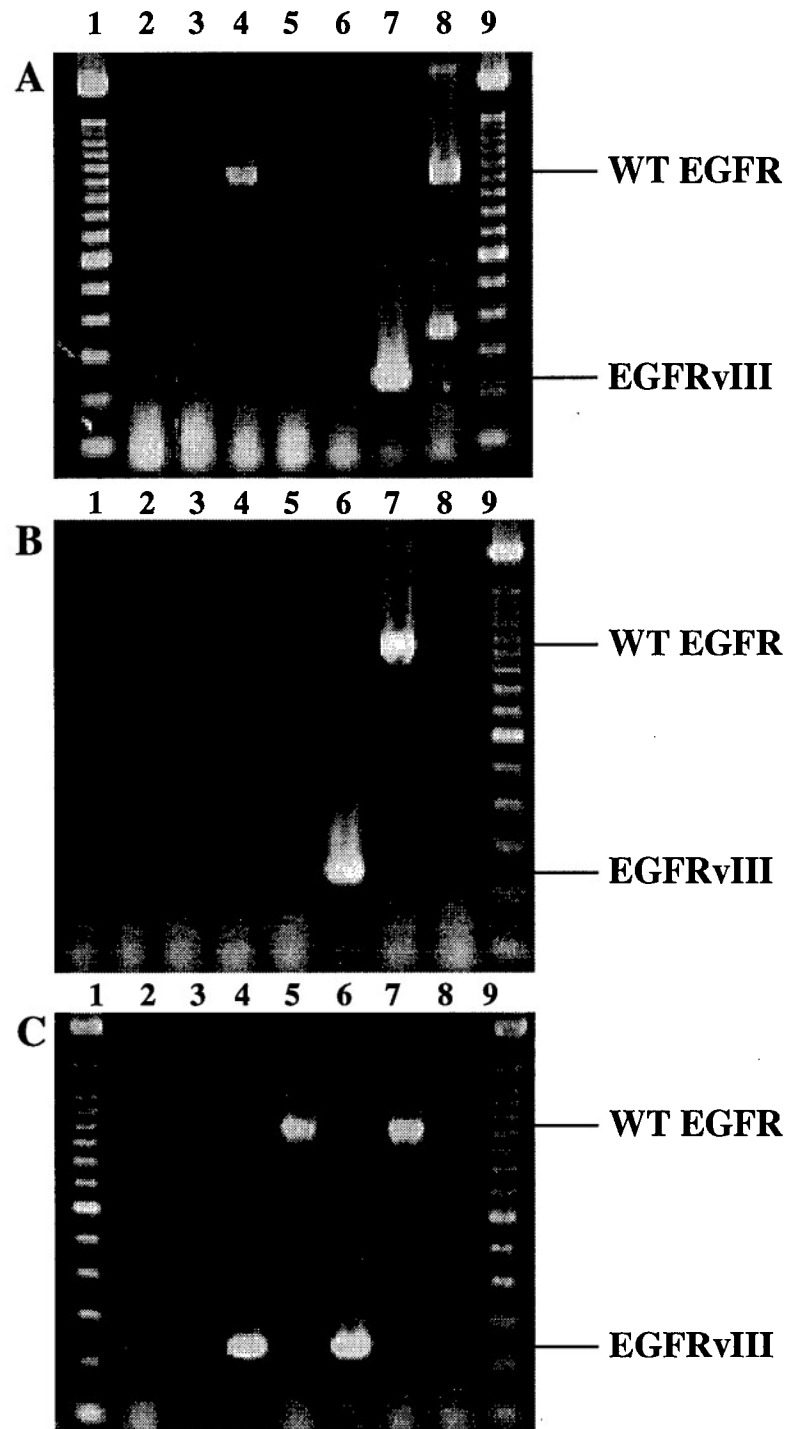
# **Production of Adenovirus Transducing EGFRvIII cDNA**

## Introduction

Characterization of the antibodies bispecific for EGFRvIII and CD3 $\epsilon$  will require functional testing using cytotoxic T lymphocyte (CTL) assays in which naive effector cells are incubated with non-major histocompatibility complex (MHC) matched EGFRvIII-expressing target cells. Lysis of the target cells will suggest that we have, in fact, bypassed the specificity of the T-cells in generating this cytotoxic response. RT-PCR analysis of several available human breast cancer permanent cell lines (MCF-7, MDA-MA-231, MDA-MA-361, MDA-MA-435, DU4475, SKBR3, ZR75-1, and T47D) demonstrates that none express the EGFRvIII tumor antigen (Figure 5.1), despite its high prevalence in primary breast tumors. Therefore, it will be necessary to construct EGFRvIII expressing breast cancer target cells to test the efficacy of the bispecific antibody construct.

Originally, I planned to generate EGFRvIII-expressing target cells by transfection of EGFRvIII cDNA, a process we have successfully accomplished in murine cell lines. However, attempts to obtain stable EGFRvIII transfectants in these breast cancer cell lines, using various transfection methods or commercially available transfection agents (calcium phosphate precipitation method, lipofectamine, Transfectam<sup>TM</sup>, and DOTAP) were unsuccessful. Furthermore, production of stable EGFRvIII-expressing cell lines using retroviral EGFRvIII constructs, which has resulted in very high level expression in other cell types, was unsuccessful in breast cancer cells, as the cells lost expression of EGFRvIII rapidly with repeated passaging. Based on these numerous unsuccessful attempts, we conclude that EGFRvIII is likely to be detrimental to survival of human breast cancer cells *in vitro*. This conclusion is supported by the observation that expression of variant forms of EGFR by human gliomas is lost over time with *in vitro* culture, necessitating the propagation of tumors known to express these variant forms of EGFR as xenografts in

**Figure 5.1: RT-PCR analysis of EGFRvIII mRNA expression in breast cancer cell lines.** mRNA was extracted from cultured breast carcinoma cell lines and subjected to RT-PCR. The cell lines studied included T47D (lane 2, panel A), ZR75-1 (lane 3, panel A), SKBR3 (lane 4, panel A), MCF-7 (lane 5, panel A), MDA-MB-231 (lane 2, panel B), MDA-MB-361 (lane 3, panel B), MDA-MB-435 (lane 4, panel B), and DU4475 (lane 5, panel B). Positive controls included glioma xenograft D256, expressing EGFRvIII (lane 7, panel A; lane 6, panels B and C) and glioma xenograft NR6W, expressing wild-type EGFR (lane 8, panel A; lane 7, panels B and C). Additional positive controls shown in panel C included the HC cell line transfected with EGFRvIII (lane 4); and the A431 cell line known to express high levels of wild-type EGFR (lane 5). The mouse fibroblast 3T3 cell line, which does not express either form of human EGFR (lane 3, panel C) is shown as a negative control. Additional negative controls lacking template were loaded in lanes 6 (panel A) and 8 (panels B and C) and negative controls for the reverse transcription reaction were loaded in lanes 1 (panel B) and 2 (panel C). 100 base pair markers are shown in lanes 9 (panels A, B and C) and 1 (panels A and C). No specific products corresponding to EGFRvIII (230 bp) are seen in any lanes containing breast cancer cell lines.



immunodeficient mice (Bigner, 1990). Therefore, we decided to create an EGFRvIII-producing adenovirus for use in these experiments. Adenoviral constructs efficiently infect epithelial cells, transducing genes with close to 100% efficiency. An adenoviral construct can be used to infect target cell lines for CTL assays, creating cells expressing high levels of EGFRvIII within 6 to 48 hours after infection. In addition, an EGFRvIII-transducing adenoviral construct is likely to have broader applications, such as potential use for active anti-EGFRvIII immunotherapeutic protocols, as discussed later in this chapter. Thus, this reagent will be very useful in our lab and others for studies involving the EGFRvIII tumor antigen.

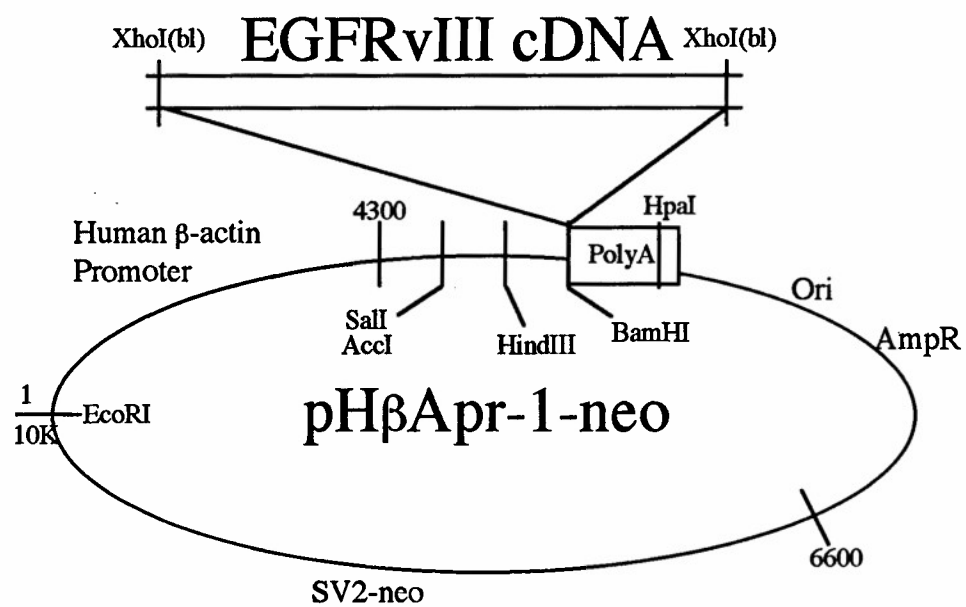
## **Results**

The following overall strategy for generation of recombinant EGFRvIII-transducing adenoviruses was as followings: First, a CMV-driven promoter and enhancer, the EGFRvIII sequence, and a stabilizing poly-adenylation sequence were assembled in the BSII cloning vector (Figure 5.6). The assembled sequence was then transferred into the pAdAscL- $\Delta$ pol adenoviral shuttle vector (Figure 5.8). Recombinant EGFRvIII-expressing virions were generated by standard methods, as described in chapter 2 (Figure 2.1).

### **Isolation of the EGFRvIII Sequence**

The EGFRvIII sequence we used was derived from pH $\beta$ Apr-1-neo-EGFRvIII, an expression vector constructed in the laboratory of our collaborator, Dr. Darell Bigner (Figure 5.2). Removal of the EGFRvIII sequence from pH $\beta$ Apr-1-neo-EGFRvIII was complicated by the fact that the XhoI sites originally flanking the cDNA sequence had been abolished during blunt end cloning into the BamHI site in the vector. A unique SalI restriction site was present in the multicloning region (MCR) 5' of the EGFRvIII sequence;

**Figure 5.2: Derivation of EGFRvIII cDNA sequence and cloning strategy.** The pH $\beta$ Apr-1-neo expression vector is driven by the beta-actin promoter, derived from a 4.3 kB EcoRI-AluI fragment from the human  $\beta$ -actin gene isolate p14T $\beta$ -17 (Leavitt, 1984; Ng, 1985). In addition the vector contains a portion of the pST64 multicloning region (MCR), a fragment derived from pcDV1 (Okayama and Berg, 1983) containing the pBR322 Ampicillin resistance gene, bacterial origin of replication, and the SV40 late region polyadenylation signal, and the PvuII-EcoRI fragment from pSV2-neo (Southern and Berg, 1982) containing the bacterial neo gene linked to the SV40 origin of replication and early promoter. To generate pH $\beta$ Apr-1-neo-EGFRvIII, an XhoI fragment containing the EGFRvIII cDNA sequence (Genbank #XOO588) was cloned into the BamHI site in the polylinker sequence of pH $\beta$ Apr-1-neo. The EGFRvIII sequence was removed from this vector by restriction enzyme digestion with SalI, which cut in the multi-cloning region upstream of the start site, and the enzyme HpaI, which cut in the middle of the SV40 late region polyadenylation signal.





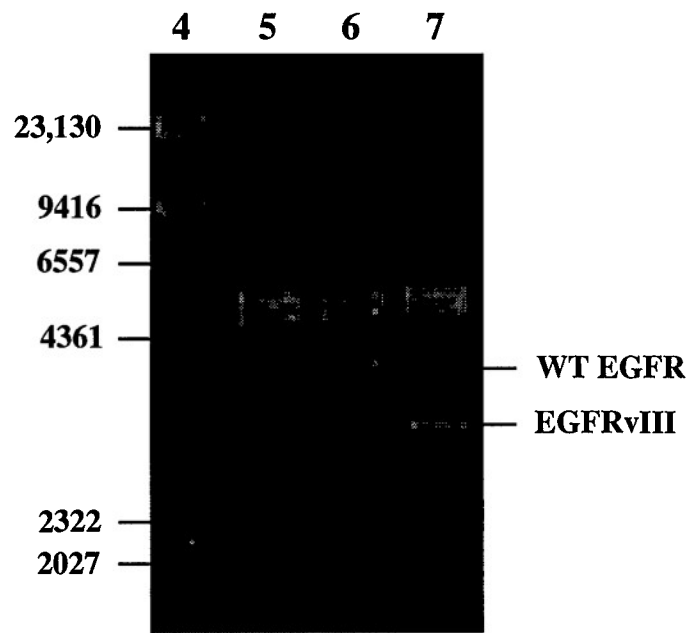
however, no unique restriction enzyme sites existed 3' of the sequence, as BamHI was the last enzyme restriction site in the MCR. Therefore, to remove the EGFRvIII cDNA sequence from the pH $\beta$ Apr-1-neo-EGFRvIII vector, the vector was digested with Sall and HpaI (which cut in the middle of the SV40 late region polyadenylation signal sequence 3' of the EGFRvIII) (Figure 5.3 A and B). Digestion of the pH $\beta$ Apr-1-neo-EGFRvIII vector with Sall and HpaI actually cut the vector into several fragments, due to the presence of additional restriction sites not recorded on our map. The size of the fragment containing the EGFRvIII sequence was unclear, since no information was available regarding the length of sequence upstream of the EGFRvIII cDNA included in the original XhoI fragment used to construct pH $\beta$ Apr-1-neo-EGFRvIII. Therefore, Southern blot analysis was performed to confirm that the fragment of approximately 4 kB contained the EGFRvIII sequence. A labeled probe specific for EGFR sequences did detect the 4 kB band, demonstrating that this Sall-HpaI fragment contained the EGFRvIII sequence (Figure 5.3C).

#### **Construction of (BSII+P/E+pA)-EGFRvIII vector**

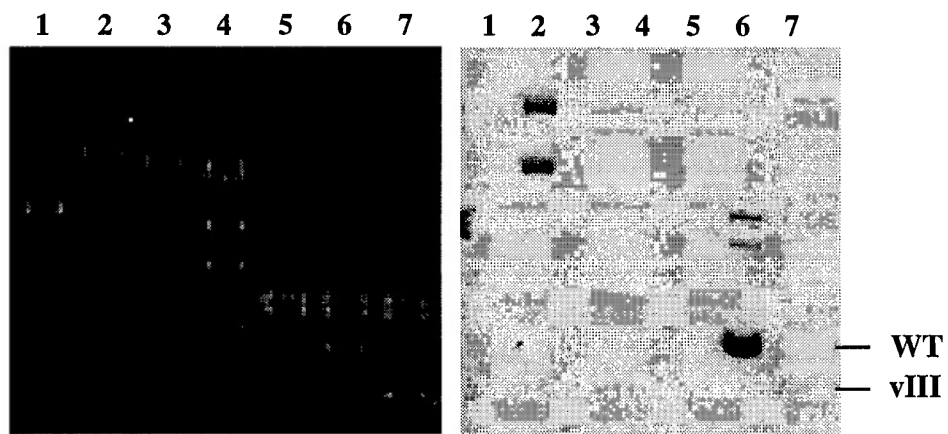
The adenovirus shuttle plasmid, pAdAscL- $\Delta$ pol, was designed so that digestion of the shuttle plasmid with AscI would allow insertion of a compatible BssHII fragment from Bluescript II (BSII) (see Figure 5.8). However, neither the shuttle plasmid nor BSII contain a eukaryotic promoter or enhancer to drive expression of the gene of interest. Thus, I cloned the following into BSII: the CMV immediate-early promoter and enhancer (CMV P/E) derived from PC1-neo, the EGFRvIII sequence, and the SV40 late region polyadenylation signal sequence 3' of the HpaI site (SV40 3'PA), also derived from PC1-neo (Figure 5.4).

To obtain the CMV P/E, PC1-neo was first cut with BglII and gel purified. The linearized vector was then reacted with T4 polymerase to create blunt ends, followed by

**Figure 5.3: Isolation of the EGFRvIII coding sequence for cloning by restriction enzyme digestion and confirmation of the correct sequence by Southern blot.** The vectors pH $\beta$ Apr-1-neo-EGFRvIII and pH $\beta$ Apr-1-neo-WtEGFR were digested with the restriction enzymes Sall and HpaI to remove the wild-type and variant EGFR sequences from these vectors. Digested fragments were analyzed on a 1.3% agarose gel, as shown in panels A and B. Lanes were loaded as follows: (panel B) - lane 1, uncut pH $\beta$ Apr-1-neo vector; lane 2, uncut, pH $\beta$ Apr-1-neo-WtEGFR vector; lane 3, uncut pH $\beta$ Apr-1-neo-EGFRvIII vector; (panels A and B) - lane 4, Lambda DNA/HindIII fragment markers; lane 5, digested pH $\beta$ Apr-1-neo vector; lane 6, digested pH $\beta$ Apr-1-neo-WtEGFR vector; and lane 7, digested pH $\beta$ Apr-1-neo-EGFRvIII vector. The low molecular band indicated in lane 7 (panel A) was presumed to contain the EGFRvIII cDNA sequence, based on an estimated MW of approximately 4 kB for the Sall-HpaI fragment containing EGFRvIII, in addition to the fact that this band was not present in the lane containing vector alone or in the lane containing wild-type EGFR-digested vector. In addition, a slightly larger unique band, presumed to contain the larger wild-type EGFR cDNA sequence, was noted in lane 6. To confirm that this sequence used for further cloning steps was EGFRvIII, the agarose gel shown in panel B was transferred to a Nylon membrane and probed for EGFR-containing sequences by Southern blot analysis (panel C). The probe used in this experiment was strongly reactive with wild-type EGFR sequences, as seen in lanes 2 and 6 containing uncut and cut pH $\beta$ Apr-1-neo-WtEGFR vector. However, only 20% of the probe was specific for sequences present in EGFRvIII (the remaining 80% represented sequences removed in the vIII deletion). Reactivity of this probe was not seen with the EGFRvIII cDNA present in the uncut vector (lane 3). Nevertheless, faint reactivity was noted with the low molecular weight band in lane 7, confirming that this digestion fragment contained the EGFRvIII sequence.



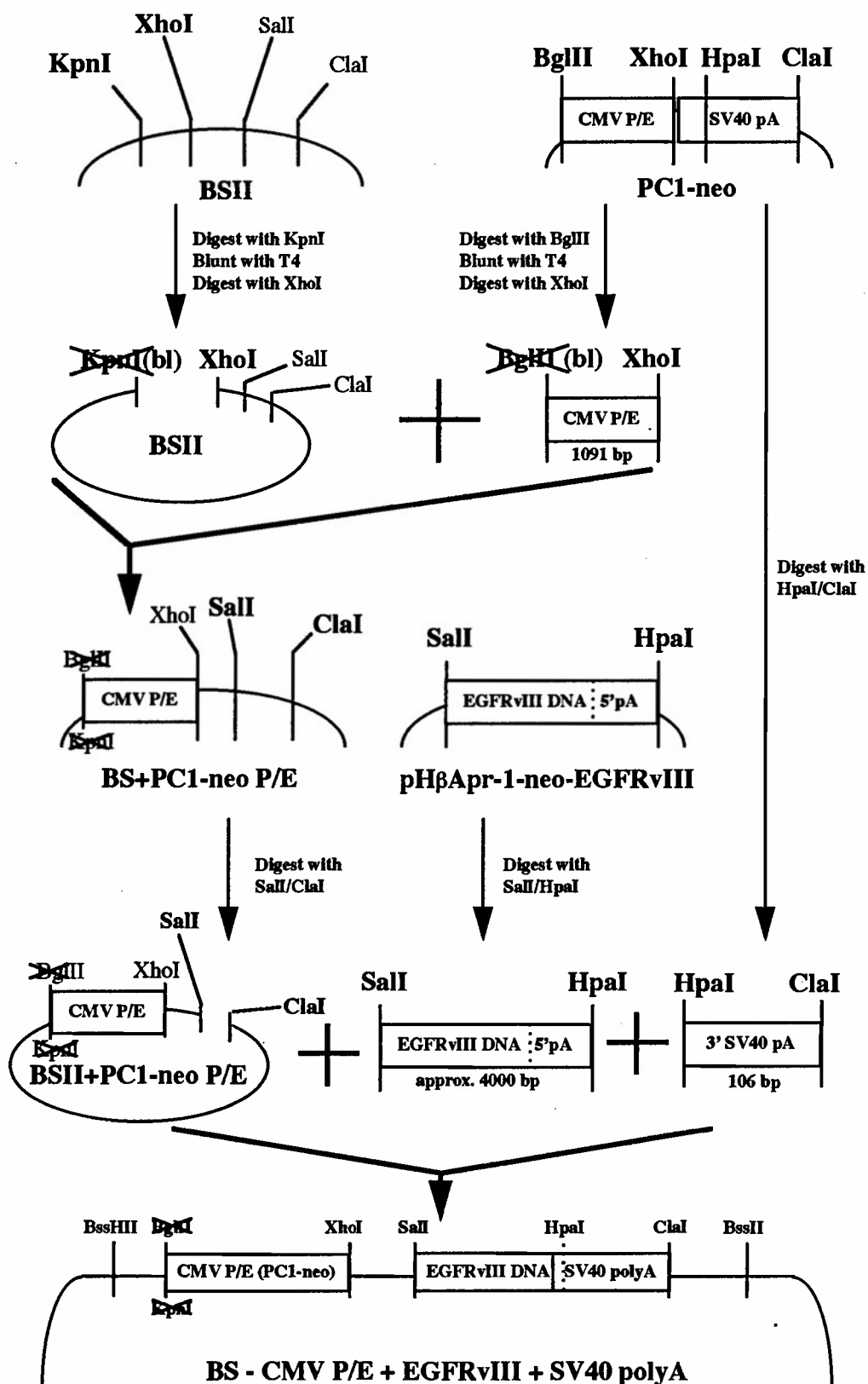
A



B

C

**Figure 5.4: Diagram of cloning strategy - insertion of CMV promoter and enhancer, polyadenylation signal, and EGFRvIII cDNA sequences into the Bluescript II cloning vector.** Sequences required for the adenoviral expression of EGFRvIII were initially assembled in the BSII cloning vector, as described in the text. Restriction enzyme sites obliterated by "blunting" with T4 polymerase are indicated with an X and the designation (bl).



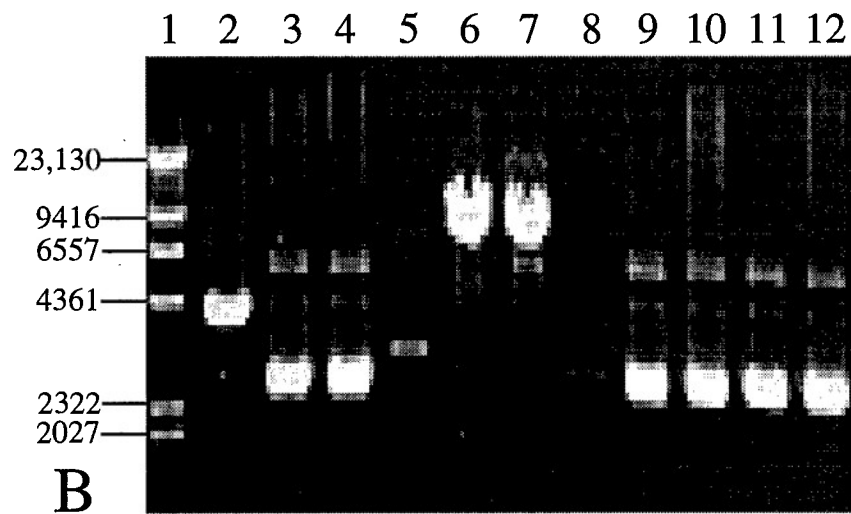
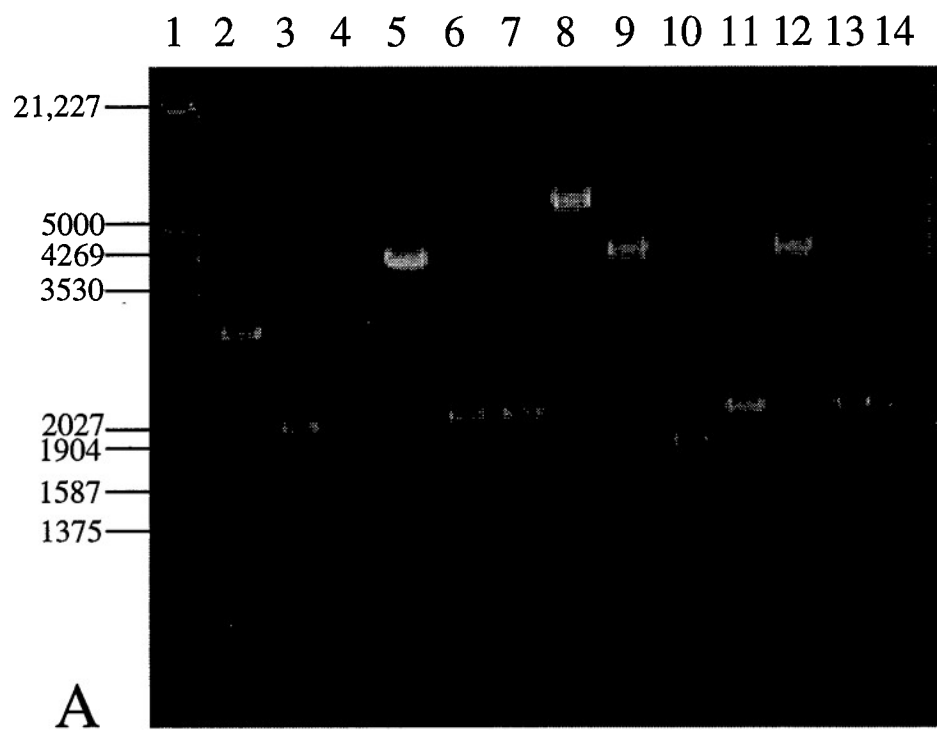
digestion with XhoI and gel purification of the 1091 bp fragment. Similarly, BSII was prepared for insertion of the CMV P/E by digestion with KpnI, gel purification, reaction with T4 polymerase, digestion with XhoI, and gel purification of the 2961 bp linearized vector. One clone containing the CMV P/E insert was selected (BSII+P/E#3) for further manipulation (Fig 5.5A).

To obtain the SV40 3'PA, PC1-neo produced in Dam-negative DM1 bacteria was digested with HpaI and ClaI, and the 106 bp fragment was gel purified. The BSII+P/E#3 vector was digested sequentially with SalI and ClaI, followed by incubation with calf intestinal phosphatase (CIP) to prevent religation of the vector, and gel purification of the linearized vector. A three fragment ligation was performed, in which the EGFRvIII SalI/HpaI fragment and the HpaI/ClaI PC1-neo fragment containing the SV40 3'PA were inserted into the BSII+P/E vector at the SalI and ClaI sites. Two good clones were obtained from this ligation and transformation (Fig 5.5B).

Linearization of the shuttle plasmid is important for efficient recombination with the adenoviral genome, and none of the four linearization sites (NheI, EcoRI, BspHI, or AseI) in the adenoviral shuttle vector was absent from the sequence I planned to insert (EGFRvIII + promoter/enhancer + polyadenylation signal) into the shuttle vector. Thus, I redesigned my cloning strategy to remove the AseI restriction enzyme site from the BSII-EGFRvIII (Figure 5.6).

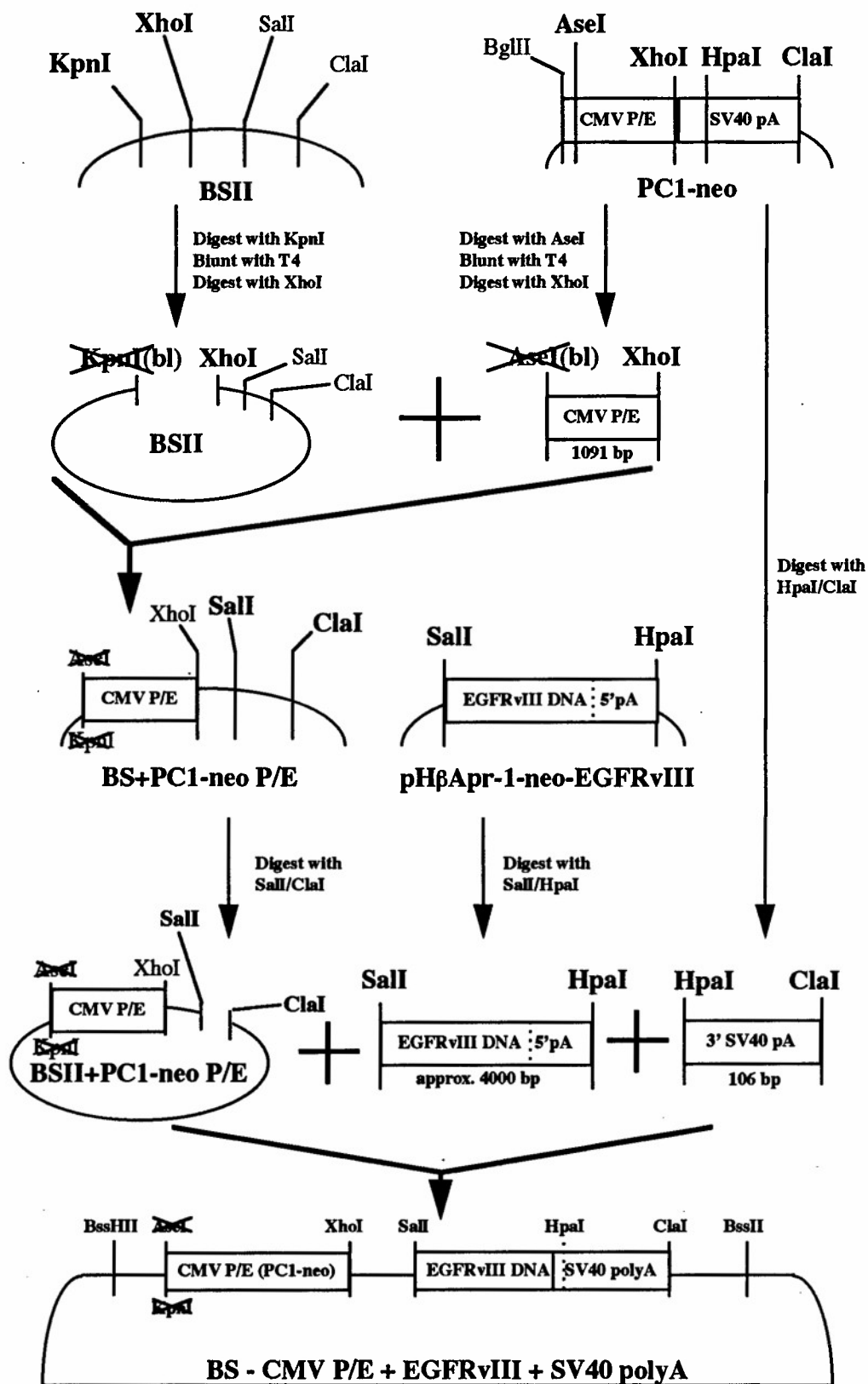
Again, the CMV I-E P/E was isolated from PC1-neo, but the 5' site restriction enzyme site used was AseI, rather than BglII. Following digestion with AseI, the 4243 bp fragment was gel purified, reacted with T4 polymerase to blunt the ends, and digested with XhoI. The 931 bp fragment containing the CMV P/E was gel purified and ligated into BSII, into the KpnI(blunt)/XhoI sites, as described above, except that the linearized BSII vector was reacted with CIP prior to the ligation to increase the likelihood of obtaining clones containing insert. Eight good clones were obtained, and these were

**Figure 5.5: Assembly of CMV promoter and enhancer, EGFRvIII sequence, and polyadenylation signal in Bluescript II cloning vector.** Panel A: The CMV promoter and enhancer were inserted into the BSII cloning vector, as described in the text and diagrammed in Figure 5.4. Twelve clones derived from this ligation were analyzed on a 1.3% agarose gel to identify constructs containing the correct insert. Clones #1-12 linearized by digestion with XhoI (lanes 3-14) were compared in size with linearized BSII vector (lane 2). Lambda DNA/EcoRI+HindIII markers are shown in lane 1. Three clones, #3, 7, and 10 (lanes 5, 9, and 12), were the appropriate size (BSII + the P/E insert was calculated to be 4052 bp, compared with BSII vector alone, 2961 bp). Panel B: Restriction fragments containing the EGFRvIII sequence and the 3' end of the poly A sequence were ligated into the BSII+P/E construct, as described in the text and diagrammed in Figure 5.4. Ten clones derived from this ligation were analyzed on a 1% agarose gel to identify constructs containing the correct insert. Clones #1-10 linearized by digestion with Sall (lanes 3-12) were compared in size with linearized BSII+P/E (lane 2). Lambda DNA/HindIII markers are shown in lane 1. Two clones, #4 and 5 (lanes 6 and 7), were the appropriate size (the construct consisting of BSII+P/E + EGFRvIII insert + polyA insert was estimated to be approximately 8000 bp, compared with BSII+P/E vector alone, 4052 bp).



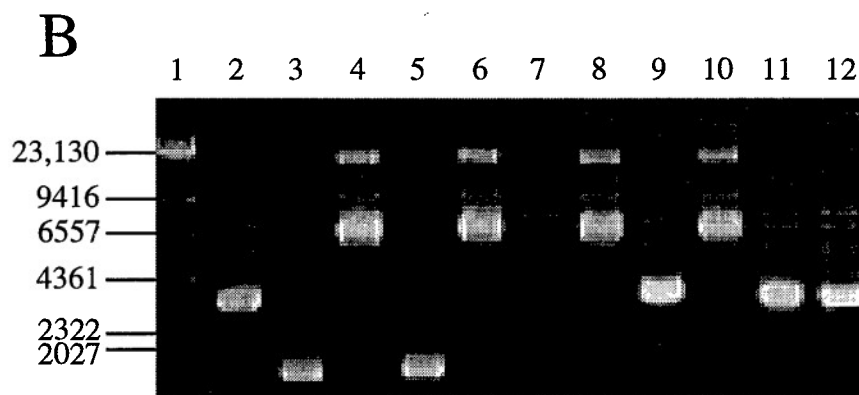
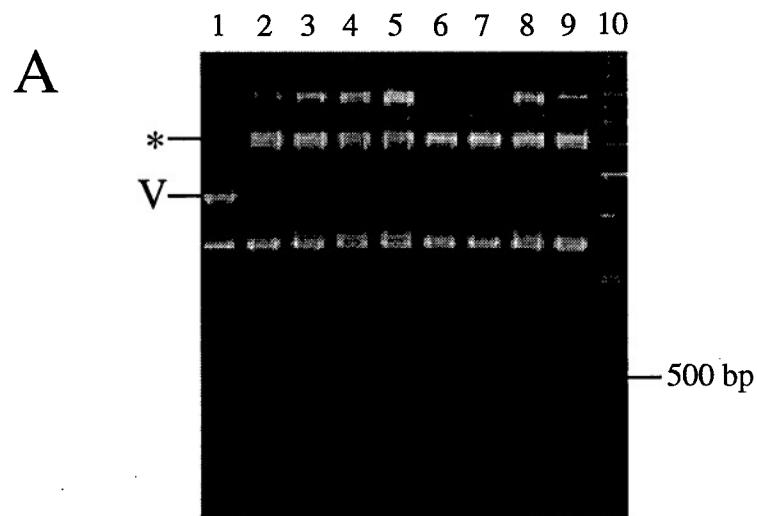


**Figure 5.6: Revised diagram of cloning strategy - insertion of CMV promoter and enhancer, polyadenylation signal, and EGFRvIII cDNA sequences into Bluescript II cloning vector, with elimination of AseI site.** A unique AseI site was required in the adenoviral shuttle vector in order to linearize the construct for co-transfection with Ad DNA and generation of a recombinant EGFRvIII-transducing virus. Thus, the strategy for assembly of the P/E, EGFRvIII, and polyA sequences in the BSII cloning vector was altered to eliminate an AseI site from the CMV promoter/enhancer sequence, as described in the text. Restriction enzyme sites obliterated by "blunting" with T4 polymerase are indicated with an X and the designation (bl).



**Figure 5.7: Assembly of CMV promoter and enhancer, EGFRvIII sequence, and polyadenylation signal in Bluescript II cloning vector, with ablation of AseI site.**

**Panel A:** The CMV promoter and enhancer were inserted into the BSII cloning vector, as described in the text and diagrammed in Figure 5.6, this time using the AseI site at the 3' end of the PC1-neo P/E to remove this fragment. Eight clones derived from this ligation were analyzed on a 1.5% agarose gel to identify constructs containing the correct insert and ensure that the blunted AseI site was eliminated. Clones #1-8 were thus digested with AseI (lanes #2-9) and compared to AseI-digested BSII vector (lane 1). 500 bp markers are shown in lane 10. AseI cut the BSII vector three times, but it was not expected to cut the inserted sequence. All ten clones contained an insert of the appropriate size for correct insertion of the P/E sequence. The fragment of BSII containing the P/E insert, (indicated in the figure by an asterisk) was calculated to be 2598 bp, compared with the 1667 BSII fragment without insert (indicated by V). **Panel B:** Restriction fragments containing the EGFRvIII sequence and the 3' end of the poly A sequence were ligated into the BSII+P/E construct, as described in the text and diagrammed in Figure 5.6. Ten clones derived from this ligation were analyzed on an agarose gel to identify constructs containing the correct insert. Clones #1-10 (lanes 2-11) were compared in size with the BSII+P/E vector (lane 12). Lambda/HindIII markers are shown in lane 1. Clones 3, 5, 7, and 9 (lanes 4, 6, 8, and 10) were all identical in size and appropriately larger than the BSII+P/E vector; thus, these four clones were presumed to contain the correct insert.



digested with *AscI* to confirm deletion of the *AscI* site derived from PC1-neo (Figure 5.7A). The new BSII+P/E Clones #1 and #5 were selected for insertion of the EGFRvIII and SV40 3'PA sequences, as described previously. Four clones of the appropriate size were obtained (Fig 5.7B).

#### **Construction of AV-EGFRvIII vector**

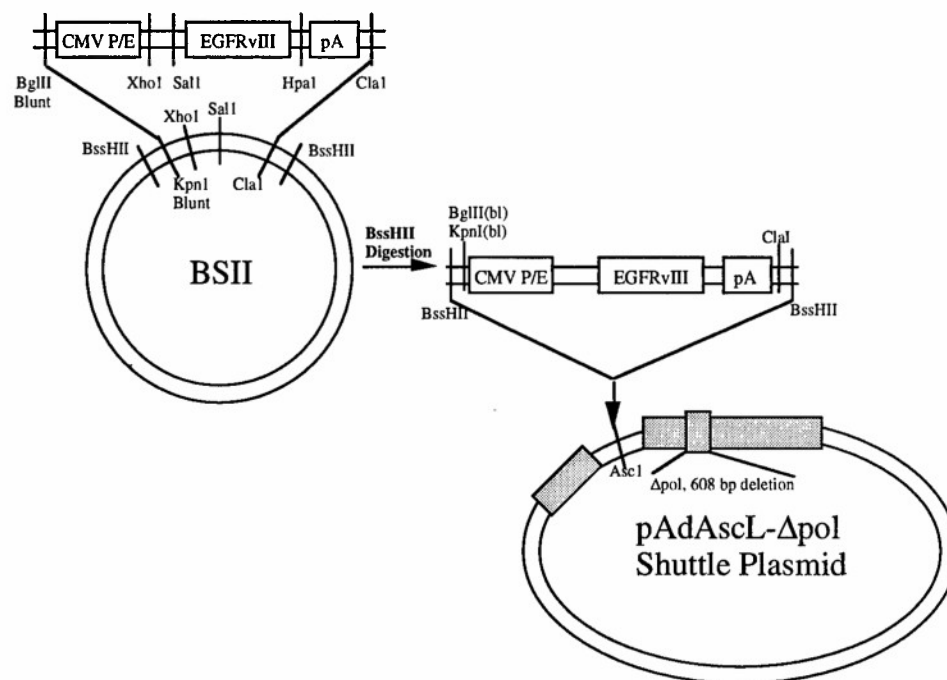
The adenoviral shuttle vector pAdAscL- $\Delta$ pol was digested with *AscI*, reacted with CIP, and gel purified. One good clone containing the appropriate insert sequence, (BSII+P/E+pA)-EGFRvIII #5, was digested with *Bss*III (which has compatible ends with *AscI*), and the 4500 bp fragment was gel purified and ligated into the adenoviral shuttle vector at the *AscI* site (Figure 5.8A). The ligation reaction was transformed into *E.coli* XL10-gold ultracompetent cells, designed to support the growth of large plasmids, and three good clones were obtained (Figure 5.8B). AV-EGFRvIII#3 was then linearized with *AscI* for use as a recombination substrate with the adenoviral genomic DNA.

#### **Production of recombinant adenoviral genomes.**

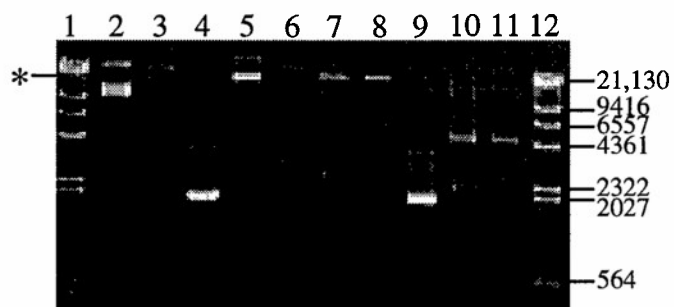
After successful insertion of the EGFRvIII sequence into the adenovirus shuttle plasmid, the shuttle plasmid was cotransfected with restriction enzyme digested non-infectious viral genomic plasmid DNA, and recombinants were selected by their ability to form plaques on the E1- and pol-complementing cell line B6, as described in chapter 2. Five clones were obtained, and these were screened for the presence of the EGFRvIII sequence by isolation and digestion of the genomic viral DNA (data not shown), as well as by PCR analysis (Figure 5.9A). Bands of the appropriate molecular weight were detected for two of the five clones - #3 and #5. To ensure that these both contained the complete EGFRvIII sequence encoding protein of the correct molecular weight, LP-293 cells were infected with the putative EGFRvIII-expressing Ad clones and Western blot analysis was

**Figure 5.8: Construction of adenoviral shuttle plasmid containing the EGFRvIII sequence.** Panel A: The CMV promoter and enhancer (P/E), EGFRvIII, and polyA (pA) sequences were first assembled in the BSII cloning vector, as shown in Figure 5.6. Digestion of BSII with BssHII, which flanks the multi-cloning region in the BSII vector, generates a fragment with sticky ends compatible for insertion into the unique AscI site in the pAdAscL- $\Delta$ pol shuttle vector, as diagrammed in panel A. The EGFRvIII-containing shuttle vector was then cotransfected into C-7 complementing cells along with non-infectious fragmented viral genomic DNA, as depicted in Figure 2.1. Panel B demonstrates that the assembled EGFRvIII + CMV P/E + pA fragment was successfully ligated into the adenoviral shuttle plasmid. Clones #1-10 were analyzed on a 1% agarose gel (lanes 3-12) and compared to empty shuttle vector (lane 2). Lambda DNA/HindIII markers are shown in lane 10. Clones 3, 5, and 6 (lanes 5, 7, and 8) were all identical in size and appropriately larger than the empty adenoviral shuttle vector; thus, these four clones were presumed to contain the correct insert.

A



B



performed (Figure 5.9B). One clone of the five, AV-EGFRvIII#5, produced protein which reacted with anti-EGFRvIII mAb L8A4 at the appropriate molecular weight on Western blot and was therefore presumed to be correct. This clone was expanded in culture and purified, as described in chapter 2.

**AV-EGFRvIII induces high level expression of EGFRvIII in breast carcinoma cells.**

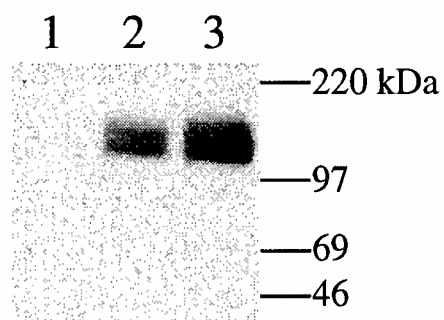
Infection of breast carcinoma cells with EGFRvIII-producing adenovirus results in very high level expression in these cells. I have demonstrated this using a variety of techniques. Anti-EGFRvIII mAb L8A4 reacts with a protein of the correct molecular weight in lysates from infected MCF-7 breast carcinoma cells by Western blot analysis (Figure 5.10A). In addition, I evaluated the expression of EGFRvIII in infected cells by flow cytometric analysis (Figure 5.10B). These data demonstrate that the infected cells do express EGFRvIII and confirm the cell surface localization of the transduced protein. Finally, immunohistochemical analysis demonstrates strong reactivity of mAb L8A4 with >75% of AV-EGFRvIII infected cells, with the majority of the remaining cells weakly reactive (Figure 5.10C). Only rare cells show no evidence of reactivity. Efficiency of adenoviral infection and EGFRvIII transduction may be enhanced by using higher titers of adenovirus. No cytopathic effects are detectable, consistent with the inability of these deleted adenoviruses to multiply in non-permissive cells not previously transfected to produce the E1 and polymerase proteins. Thus, this EGFRvIII-transducing adenovirus will serve as a very useful reagent for induction of short-term, high level expression of the EGFRvIII protein, as necessary to create target cells for tests of the bispecific antibody reagent or for use in immunotherapy protocols.



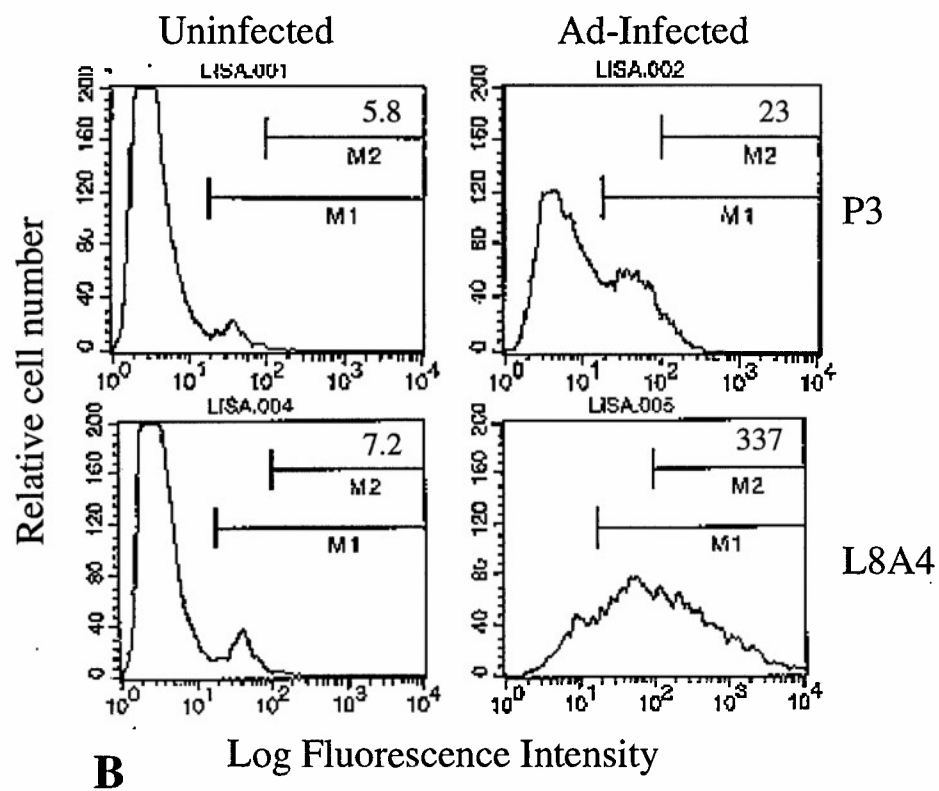
**Figure 5.9: Confirmation of EGFRvIII expression by recombinant adenoviral clones by polymerase chain reaction and Western blot analysis.** Panel A: DNA from five adenoviral clones (lanes 2-6) were analyzed by PCR and agarose gel electrophoresis for the presence of EGFRvIII sequences. Products specific for EGFRvIII DNA were detected in two clones (#3 and 5, lanes 4 and 6), suggesting that at least a portion of the EGFRvIII sequence had been incorporated into the viral genome by recombination. Positive controls included PCR analysis of vectors containing EGFR wild-type (lane 7) and vIII (lane 8) sequences. 100 bp markers are shown in lane 1, and a negative control lacking template was loaded in lane 9. In panel B, lysates from LP-293 cells infected with five AV-EGFRvIII clones (lanes 1-5) or uninfected (lane 6) were reacted with anti-EGFRvIII mAb L8A4 in Western blot assays. As a positive control, a protein lysate from the HC mouse cell line stably transfected with EGFRvIII, was loaded in lane 9. Lanes 7 and 8 contained molecular weight markers and no protein, respectively. Identical bands consistent with the 140 kDa molecular weight of EGFRvIII were detected by mAb L8A4 in both the positive control (lane 9) as well as the lane containing AV-EGFRvIII clone #5 (lane 5). No bands were detected on an identical blot using negative control antibody P3x63 (not shown). Higher molecular weight bands represent glycosylated forms of EGFRvIII and are consistently seen, along with the 140 kDa band, on Western blot (Wikstrand, 1995).



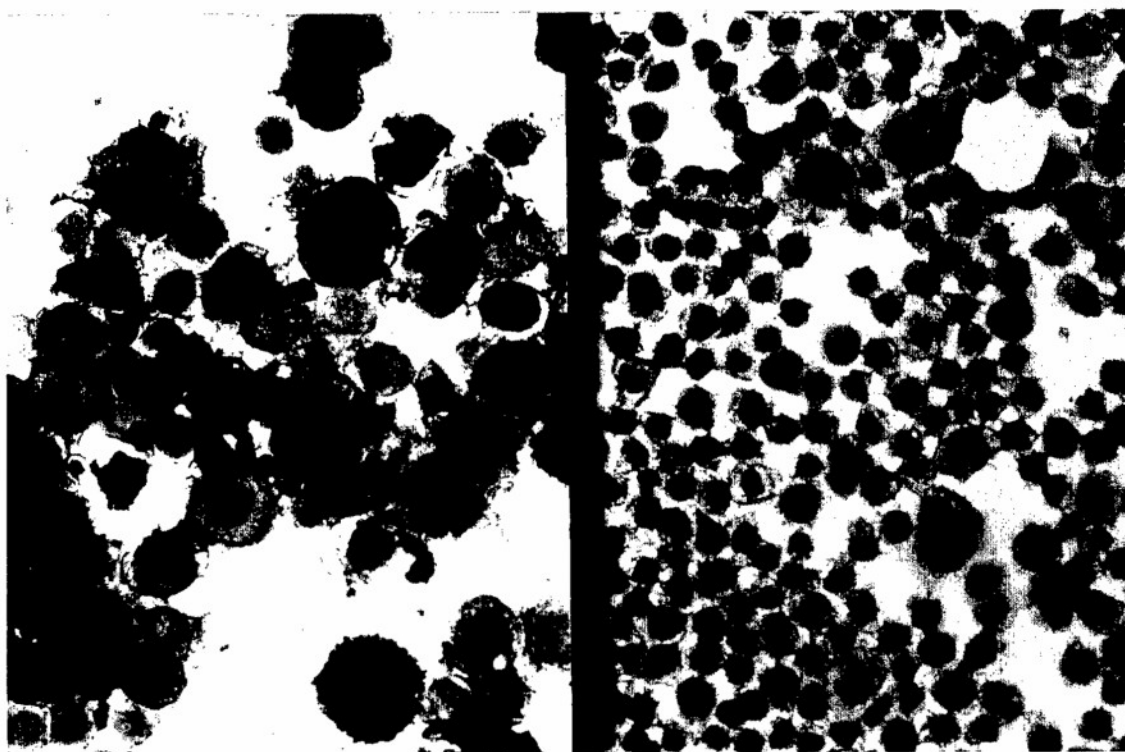
**Figure 5.10: Infection of MCF-7 breast carcinoma cells with AV-EGFRvIII results in high level expression of the variant EGFR protein by Western blot, flow cytometric and immunohistochemical analyses.** In panel A, MCF-7 cells were infected with 600  $\mu$ l (lane 2) or 1 mL (lane 3) of AV-EGFRvIII clone #5 viral preparation. The cells were harvested 46 hours following infection, and lysates were reacted with anti-EGFRvIII mAb L8A4 in Western blot assays. Bands consistent with the 140 kDa protein EGFRvIII were specifically detected in lysates from AV-EGFRvIII-infected MCF7 cells, but not in lysate from uninfected MCF-7 cells (lane 1). No bands were detected on an identical blot using negative control antibody P3x63 (not shown). The two bands represent the 140 kDa EGFRvIII species and a higher molecular weight, glycosylated species. The pattern appears different here than in Figure 5.9, as these samples were separated on a gradient gel, rather than the 7.5% gel shown in Figure 5.9. Panel B: MCF-7 cells were infected with EGFRvIII-transducing adenovirus and reacted with EGFRvIII-specific (L8A4) or negative control (P3x63) mAb followed by FITC-goat-anti-mouse Ig. Infected MCF7 cells reacted with L8A4 (bottom right panel) had a mean fluorescence of 337, compared with infected cells reacted with P3x63 negative control (top right), which had a mean fluorescence of 23. Uninfected MCF7 cells did not react with either the EGFRvIII-specific mAb (bottom left, mean fluorescence 7.2) or the negative control mAb (top left, mean fluorescence 5.8). The slightly increased mean fluorescence of infected cells reacted with negative control antibody may be due to non-specific effects of viral infection. Panel C: MCF-7 cells cultured *in situ* on glass slides were infected with 600  $\mu$ l of AV-EGFRvIII clone #5 viral preparation and analyzed immunohistochemically for expression of EGFRvIII protein. Infected cells were strongly reactive with anti-EGFRvIII mAb L8A4 (left panel), in comparison with non-infected cells (right panel).



**A**



C



## Discussion

I have constructed a recombinant adenovirus which efficiently transduces EGFRvIII cDNA, creating high level transient expression of this protein in breast cancer cells. This EGFRvIII delivery method will also work in any other human cell lines which express an adenovirus receptor, which includes most non-hematopoietic cells, as well as many cells derived from other species. Thus, this EGFRvIII adenovirus is likely to be useful, not only for evaluation of the bispecific antibody construct, but for many other applications, including study of the EGFRvIII tumor antigen in breast cancer cells *in vitro*, as well as for applications studying the interaction between the immune system and the EGFRvIII tumor-associated antigen *in vivo*. In addition, this construct may be a useful agent for active immunotherapeutic protocols targeting EGFRvIII-expressing tumors.

Previous work in our laboratory has demonstrated that host immune responses against a specific tumor antigen can be effectively induced by administration of an allogeneic cellular vaccine genetically modified to express that tumor antigen (Ashley, 1997). In this study, stably transfected allogeneic cells expressing the EGFRvIII tumor antigen were injected into mice bearing EGFRvIII-expressing tumors. Class I-restricted (cytotoxic), antigen-specific responses were generated in these animals, with a significantly improved long-term survival rate, from 0% untreated to 50% treated. As previously discussed, induction of an immune response against a tumor-associated antigen is technically challenging for many reasons, and allogeneic cellular vaccination may provide a means of bypassing these problems. Although the mechanistic details are poorly understood at this time of how immune responses are induced by allogeneic cellular vaccines, it is clear that exposure of the host immune system to an antigen

(EGFRvIII), in an environment of allogeneic immune induction is a much stronger immunogenic stimulus than exposure of that same antigen in a context lacking the foreign MHC factor. Thus, genetic manipulation of allogeneic cells to express tumor-associated antigens for presentation to and stimulation of the host immune system is a very promising active immunotherapeutic approach.

Our previous study (Ashley, 1997) demonstrated the efficacy of this allogeneic tumor vaccine approach using stably transfected EGFRvIII-expressing cells. The use of stably transfected cells has several advantages, including that the transfection of allogeneic cells with a relevant tumor antigen provides a homogeneous population of cells, all of which (if cloned) express that antigen. The disadvantages to this approach are that tumor antigens such as EGFRvIII are not expressed in every patient's tumor, and more importantly, that antigen-loss variants are likely to be selected for by this therapy, even if the tumor does express the antigen initially. A solution to this problem would be administration of a "cocktail" of tumor antigens, potentially designed specifically for the molecular defects found in an individual patient's tumor, in the context of an immunostimulatory allogeneic cellular vaccine. In order for individually designed tumor vaccines to be technically and financially feasible, the components of the vaccine should be available prepared ahead of time, easy to combine, and require little laboratory manipulation prior to administration to the patient. Stable transfection with a cocktail of tumor antigens does not meet these requirements for feasibility. However, an alternative approach is to infect allogeneic cells with a mixture of tumor-antigen-expressing adenoviridae. Very high level expression in a majority of the cell population is achieved rapidly (within 24 hours) following infection. Thus, vaccine therapy could be potentially administered within a day of determining which antigens are expressed on a patient's tumor and thus would be good immunotherapeutic targets.

Much of the recent work involving adenoviral gene delivery has focused on making recombinant adenoviruses less immunogenic for the purpose of maintaining long-term expression of a transduced protein, such as dystrophin. In this case, the anti-viral host immune response is currently the main limitation on the effectiveness of this type of gene therapy (Amalfitano, 1998). The immune response not only limits the duration of expression of the transgene, but also prevents effective repeated administration of the recombinant adenovirus. However, in gene therapy for cancer, the immunogenic response to the adenovirus would actually be beneficial (Descamps, 1996). In fact, different adenoviral vector systems have been generated in which the immunogenicity is enhanced. For example, vectors lacking the adenoviral E3 gene, important in host immune evasion and thus persistence *in vivo*, are ideal for use in vaccination protocols (Hitt, 1995). The vector system we have used is a second generation vector designed such that its immunogenicity is minimized, and infected cells persist *in vivo* for up to 6 weeks in mice (A. Amalfitano, unpublished data). If required, insertion of the cassette, containing the EGFRvIII sequence with its promoter, enhancer, and polyadenylation signal, into a more immunogenic vector would be a simple matter of transferring the pre-assembled sequence from the BSII cloning vector. However, this manipulation is unlikely to be required for use in allogeneic tumor cell vaccines. The immunogenicity of these vaccines would derive from expression of the EGFRvIII antigen in association with foreign MHC molecules on the infected cells, irrespective of whether the immune system is concurrently responding to the low level expression of adenoviral proteins. Thus, the EGFRvIII-transducing adenovirus will not only be useful in testing the bispecific antibody construct, but it will also potentially be an effective reagent for use in active immunotherapy.



## Summary

In summary, I have produced a recombinant E1<sup>-</sup>/pol<sup>-</sup> deleted adenovirus capable of efficient transduction of cDNA for the EGFRvIII tumor-associated antigen into breast cancer and other cell types. This construct should prove useful for testing the efficacy of bispecific antibody constructs, as well as having a potential for use in active immunotherapy protocols directed against EGFRvIII.

## **Chapter 6**

### **Summary**

Prevention of metastatic breast cancer is limited to early detection and surgical resection of a localized tumor. Treatment for advanced breast cancer is often unsuccessful due to micrometastasis of the tumor prior to therapy. Thus, new methods to treat and prevent metastatic breast cancer are sorely needed.

Prevention and treatment of breast cancer is advanced via an improved understanding of breast cancer biology. We have developed immunological reagents against the inherited breast cancer susceptibility gene product BRCA2. In addition, we have shown that this protein is expressed at high levels in thymic epithelium, skin, and squamous carcinoma, suggesting a potential role in differentiation. Elucidation of the role this protein plays in normal breast biology may help us to understand why tumorigenesis occurs in hereditary cases, and these findings may also be applicable to non-inherited breast cancer.

EGFRvIII, a variant form of the epidermal growth factor receptor, is found in a large percentage of breast tumors and thus may be a viable target for immunotherapy. I have developed a bispecific antibody against both EGFRvIII and a T-cell activation antigen to redirect the cytotoxic response of T-cells with a broad range of specificities against breast cancer cells that express EGFRvIII. In addition, I have developed an EGFRvIII-transducing adenovirus which will be useful in active immunotherapeutic protocols targeting the EGFRvIII tumor antigen.

# **Chapter 7**

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### **Education:**

- Medical School**      **Duke University School of Medicine, Durham, NC ('92-'99)**  
- MD candidate, anticipated graduation 5/99  
- Honors awarded in Medicine and Family Medicine
- Graduate School**      **Duke University Graduate School, Durham, NC ('94-'98)**  
- PhD, Department of Pathology  
- Honors awarded in Comprehensive Immunology;  
Medical Immunology, Tumor Immunology
- College**      **Amherst College, Amherst, MA ('87-'91)**  
- B.A. in Biology  
- Cumulative average: A-
- The University of New South Wales, Sydney, AU (Spring '90)**  
- Completed coursework equivalent to one semester at  
Amherst College; honors level of Distinction

### **Honors and Awards:**

- 1996-1999      Predoctoral peer-reviewed training grant, entitled "An Immunotherapeutic Approach to the Treatment and Prevention of Breast Cancer, Based on Epidermal Growth Factor Receptor Variant, Type III" awarded by the U.S. Army Medical Research and Materiel Command:  
Total amount funded = \$60,737
- 1992-1999      Medical Scientist Training Program, Duke University School of Medicine
- 1991      Magna Cum Laude, Amherst College
- 1991      Oscar E. Schotte Scholarship Prize (given to one senior Biology major at Amherst College)
- 1987      National Merit Commendation
- 1984-1987      Math, Science, French, Latin, and Cum Laude High School Honors Societies, The Hockaday School, Dallas, TX

**Certified:**      United States Medical Licensing Exam: Step I - 1995, Step II - 1994

## Research Training:

- 1/95-8/98      Tumor Immunopathology & Cellular Immunology:  
**Dr. Laura Hale**, Department of Pathology,  
Duke University School of Medicine.  
Research leading to a Ph.D.; Dissertation title "Immune-Based  
Approaches to Breast Cancer Susceptibility and Treatment"
- 9/91-6/92      Keratinocyte Biology: **Dr. Peter Elias**, Dermatology Service,  
Department of Veterans Affairs Medical Center, San Francisco.  
Investigation of the effects of heparin and heparin derivatives on  
the growth and differentiation of human foreskin keratinocytes.
- 7/90-8/91      Cell Biology: **Dr. Patrick Williamson**,  
Department of Biology, Amherst College.  
Senior honors thesis: "Determining the Relationship Between Loss  
of Phospholipid Asymmetry and Vesiculation of the Human  
Erythrocyte Membrane."
- 6/88 - 8/88      Cellular Immunology: **Dr. Timothy Sullivan**,  
Division of Allergy, Southwestern Medical School.  
Study of the correlation between allergy to Mountain Cedar (as  
assessed by skin test and RAST) and HLA haplotype.

## Other Employment and Teaching Experience:

- Teaching Assistant**    Principles of Immunology, Undergraduate course, Duke U.,  
Department of Immunology (Dr. Donna Kostyu) - Spring '98
- Current Topics in Biomedical Research, Freshman seminar,  
Duke University Medical School (Dr. Dan Blazer) - Spring '97,'98
- Molecular Aspects of Disease, Graduate course, Duke University,  
Department of Pathology (Dr. Laura Hale) - Fall '97,'98
- Animal Physiology, Undergraduate course, Amherst College,  
Department of Biology (Dr. Patrick Williamson) - Spring '91
- Facilitator**            Clinical Arts, Medical school course, Duke University,  
Dept. of Family Med. (Drs. Barbara Sheline, Joe Corless) - Fall '95
- Instructor**            Preparation for the MCAT, Kaplan Test Prep, Durham, NC, taught  
biology, inorganic/organic chemistry, physics, writing - Spring '95
- Tutor**                 Introductory Biology, Amherst Dean of Students Office - Fall '90
- Phlebotomist**        Parkland Memorial Hospital, Dallas, TX - Summer '88

## Professional Society Memberships:

1991-present	Sigma Xi, National Honorary Scientific Research Society
1992-present	American Medical Association
1992-present	North Carolina Medical Society
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## Publications and Abstracts:

**Gilliam, L.K.,** Lobenhofer, E.K., Greer, P., Searce, R.M., Cirisano, F.D., Moron, A., Berchuck, A., Marks, J.R., and Hale, L.P.: BRCA2 MAb's React with Differentiating Epithelium, submitted, 1998.

**Gilliam, L.K.,** Greer, P.K., Li, J., Marks, J.R., Hale, L.P.: BRCA2 Antibodies React with Skin and Squamous Carcinomas. *American Journal of Clinical Pathology*, in press, Oct. 1998.

**Gilliam, L.K.,** Greer, P.K., Hale, L.P.: Passive Specific Cancer Immunotherapy Based on an Antibody Bispecific for Epidermal Growth Factor Receptor Variant III and CD3-epsilon. *Proceedings of the Department of Defense Breast Cancer Research Program Meeting, Era of Hope*. Vol III:943-944, 1997.

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## Extracurricular and Volunteer Activities:

Volunteer, Durham Interfaith Hospitality Network (Laura Benson) - '97-present

Choir member, Duke University Chapel Choir (Rodney Wynkoop) - '95-present

Mentor, Duke University Women's Center, Graduate Women's Mentoring Program

Volunteer, NC Rural Health Coalition (Barbara Sheline) - '94-'95

Other interests: horseback riding, backpacking, cooking Indian food, traveling, gardening, knitting, Duke University Women's Center activities (including community volunteer work, such as Habitat for Humanity)

## Biography

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Lisa Katherine Gilliam was [REDACTED]. She was raised in Dallas, Texas, where she attended the Hockaday School. Her undergraduate training took place at Amherst College in Massachusetts, where she majored in biology. She graduated from Amherst College Magna Cum Laude in May, 1991, having completed an honors thesis project in the laboratory of Dr. Patrick Williamson, studying erythrocyte membrane structure. She then spent a year in San Francisco, California, working in the laboratory of Dr. Peter Elias at the Veteran's Administration medical center, where she studied keratinocyte biology. She is currently enrolled in the Medical Scientist Training Program at Duke University. Her graduate training, under the supervision of Dr. Laura Hale in the Department of Pathology, will culminate in the completion of a Ph.D. dissertation entitled "Immune-Based Approaches to Breast Cancer Susceptibility and Treatment." Following completion of her graduate training, she will pursue a residency in Internal Medicine and plans to embark on a career in academic medicine.